JAN 30 1925

THE JOURNAL OF GENERAL PHYSIOLOGY

Founded by Jacques Loeb

EDITORS

W. J. CROZIER JOHN H. NORTHROP

W. J. V. OSTERHOUT

VOLUME VII, No. 3 JANUARY 20, 1925

PUBLISHED BIMONTHLY

AT MOUNT ROYAL AND GUILFORD AVENUES, BALTIMORE, MD.

BY THE ROCKEFELLER INSTITUTE FOR MEDICAL RESEARCH

COPYRIGHT, 1925, BY THE ROCKEFELLER INSTITUTE FOR MEDICAL RESEARCH

Entered as second-class matter November 23, 1918, at the Post Office at Baltimore, Md., under the Act of March 3, 1879.

Made in the United States of America

PUBLICATIONS OF THE ROCKEFELLER INSTITUTE FOR MEDICAL RESEARCH

THE JOURNAL OF EXPERIMENTAL MEDICINE

EDITED BY

SIMON FLEXNER, M.D. PEYTON ROUS, M.D.

The Journal of Experimental Medicine is designed to cover the field of experimental medicine. It is issued monthly, two volumes of over 600 pages each appearing in a year. Beginning with Volume XLI the subscription price per year (two volumes) is $10.00, payable in advance. Single copies cost $1.00. The price of the earlier volumes per year (two volumes) is $5.00; single copies 75 cents. Remittances should be made by draft or check on New York, or by postal money order, payable to *The Journal of Experimental Medicine*, Mount Royal and Guilford Avenues, Baltimore, Md., or Avenue A and 66th Street, New York, N. Y.

THE JOURNAL OF BIOLOGICAL CHEMISTRY

EDITED BY

STANLEY R. BENEDICT, New York, N. Y. LAFAYETTE B. MENDEL, New Haven, Conn.
H. D. DAKIN, Scarborough, N. Y. DONALD D. VAN SLYKE, New York, N. Y.

The Journal of Biological Chemistry is designed for the prompt publication of original investigations of a chemical nature in the biological sciences. It is issued monthly. Each volume consists of as many numbers as are required to make a total of approximately 800 pages. The subscription price is $5.00 per volume to subscribers in the United States and Canada, payable in advance; $5.25 to those in other countries. The number of volumes issued yearly averages four. The price of single copies varies; it is given on the back cover of each number. Remittances should be made by draft or check on New York, or by postal money order, payable to *The Journal of Biological Chemistry*, Mount Royal and Guilford Avenues, Baltimore, Md., or Avenue A and 66th Street, New York, N. Y.

THE JOURNAL OF GENERAL PHYSIOLOGY

EDITED BY

W. J. CROZIER JOHN H. NORTHROP W. J. V. OSTERHOUT

The Journal of General Physiology is devoted to the explanation of life phenomena on the basis of the physical and chemical constitution of living matter. Information regarding contributions and subscriptions is given in full on the back cover.

STUDIES FROM THE ROCKEFELLER INSTITUTE FOR MEDICAL RESEARCH

The published results of investigations conducted in the Institute, or under its grants, are republished at irregular intervals in volumes designated *Studies from The Rockefeller Institute for Medical Research*. The subscription price is $2.00 per volume, payable in advance. A special rate of $1.00 per volume is made to those who subscribe to *The Journal of Experimental Medicine*, *The Journal of Biological Chemistry*, or *The Journal of General Physiology*. Remittances should be made by draft or check on New York, or by postal money order, payable to The Rockefeller Institute for Medical Research, Avenue A and 66th Street, New York, N. Y.

SEMIANNUAL LIST

The Institute publishes a *Semiannual List* of all papers by members of the staff and those working under grants from the Institute, stating the title and place of publication of these reports. The *Semiannual List* will be sent regularly upon application.

THE ELECTRICAL CONDUCTIVITY OF PURE PROTOPLASM.

By S. C. BROOKS.

(From the Division of Pharmacology, Hygienic Laboratory, United States Public Health Service, Washington, D. C.)

(Accepted for publication, October 6, 1924.)

Measurements of the electrical conductivity of protoplasm free from interference by cell walls, vacuoles, intercellular spaces, and the like are for the first time given in this paper. Their great interest from the point of view of the study of permeability will readily be appreciated, even after due allowance is made for the fact that principles valid for one species cannot safely be generalized to include all living matter.

The organism used in the present experiments was the slime-mold, *Brefeldia maxima* (Fr.) Rost., which passes the vegetative period of its life cycle as a plasmodium creeping about in the decaying vegetable material of the swamps; when the time for spore formation approaches, the plasmodium, consisting of protoplasm containing many nuclei, flows out to the surface, forming small heaps having the consistency of very thick cream. Individual plasmodia furnish 5 to 20 cc. of an essentially pure protoplasm.[1]

Pure protoplasm of this type was placed in a U-tube of about 1 cm. diameter, the distance between the 8 mm. square platinized platinum electrodes being about 8 cm. The electrodes were sealed into glass tubes, containing the usual droplet of mercury, and the glass tubes kept in position by rubber stoppers inserted into the open ends of the U-tube. The position of the electrodes was fixed with sufficient

[1] Fixed and stained sections of plasmodia at this stage, as depicted in a paper by Harper and Dodge (Harper, R. A., and Dodge, B. O., *Ann. Bot.*, 1914, xxviii, 1) and as examined by me in the case of specimens kindly loaned by Rev. Bede Knapke show practically no vacuoles, although these appear occasionally at or near a surface on which protrusions due to already forming sporangia appear.

accuracy so that readings could be duplicated to within less than 3 per cent.

In filling the protoplasm into the U-tube a few small air bubbles were left which could not be removed without considerable stirring. This was avoided because excessive stirring was observed to lead to disorganization and liquefaction of the protoplasm. These bubbles did not in any case increase the electrical resistance by an appreciable amount, for they were never more than 2 mm. in diameter and were few in number.

The electrical resistances were measured by means of a bridge circuit with a 1 meter slide-wire provided with a 19 meter extension, and with a variable standard plug "post office box" resistance; the current was a 1,000 cycle alternating current generated by an "audio oscillator;" a telephone was used as a detector. No means of compensating for electrode capacitance was available. All measurements were at room temperature, not far from 22°C.

The resistance of water squeezed from the moss on which plasmodia were clambering was found to be 53,100 ohms; when protoplasm was filled into the same tube until it covered both electrodes the resistance was found to be 19,000 ohms. This represents a very low conductance, about equal to that of 0.00145 N NaCl, but one still greatly in excess of that of the fluid with which the protoplasm was in equilibrium. The net conductance[2] of the protoplasm of *Brefeldia* is normally about plus 180 per cent. The net conductance of red blood cells, yeast bacteria, *Chlorella*, teleost and echinoderm eggs, muscle, and of marine algæ in general, on the other hand, is usually a negative quantity; *i.e.*, their conductivity is less than that of the surrounding fluid. It is, therefore, of interest to see how *Brefeldia* plasmodium behaves under the influence of a salt concentration higher than normal.

The resistance of sea water diluted with distilled water to 1/100 of its original strength was found to be 717 ohms. The tube was then filled nearly to the level of the electrodes with protoplasm, enough of 1 per cent sea water added to more than cover the electrodes, and then worked into the protoplasm to displace the greater part of the air bubbles; the resistance was then found to be 688 ohms. It will be

[2] Brooks, S. C., *J. Gen. Physiol.*, 1922–23, v, 365.

noted that here again the resistance of the protoplasm is less than that of the surrounding fluid, although by a much smaller amount; the net conductance is here plus 4.1 per cent. This may be due to the fact that the time elapsing between the first contact with the solution and the measurement was not sufficient for the attainment of equilibrium.

The great difference in the conductance of the protoplasm in the two cases cannot be accounted for by the fact that the relatively good conductor, 1 per cent sea water, was used to displace the air bubbles in the latter experiment. Since the resistance of the protoplasm and 1 per cent sea water was less than that of the 1 per cent sea water alone, replacement of the latter by protoplasm could only decrease the resistance. In the first experiment the conductance of the protoplasm was 688/19,000 of that in the second. So low a conductance could be caused by air bubbles only if they occupied on the average more than 26/27 of the cross-sectional area. The bubbles of air certainly occupied less than 1 per cent of this area, and consequently cannot be held accountable.

The experiments therefore confirm the suggestion[2] that the conductivity of protoplasm varies with that of the surrounding fluid, thus implying permeability to electrolytes, and the existence of an equilibrium between protoplasm and environment.

The experiments here reported are admittedly of the roughest kind, and can only be considered as preliminary; but they are not only of intrinsic interest, but suggest further experiments which should be most instructive. These the writer hopes to take up at an early opportunity.

SUMMARY.

The electrical conductivity of the plasmodium of the slime-mold *Brefeldia maxima* (Fr.) Rost., which constitutes practically pure protoplasm, was found to be approximately equivalent under normal conditions to that of a 0.00145 N NaCl solution, and about 2.8 times that of the liquid in contact with which it developed.

When bathed in 1 per cent sea water, the conductivity was much increased, becoming greater than that of the surrounding fluid.

These preliminary tests are in agreement with the supposition that the protoplasm is permeable to and in equilibrium with its environment in so far as electrolytes are concerned.

This work was done at the Marine Biological Laboratory, Woods Hole, with facilities and apparatus kindly made available by the Laboratory and by Dr. Maurice Visscher, and to both the writer wishes to express his indebtedness and thanks.

STUDIES ON BIOLUMINESCENCE.

XVII. Fluorescence and Inhibition of Luminescence in Ctenophores by Ultra-Violet Light.

By E. NEWTON HARVEY.

(From the Physiological Laboratory, Princeton University, Princeton, the Nela Research Laboratories, Cleveland, and the Marine Biological Laboratory, Woods Hole.)

(Accepted for publication, October 21, 1924.)

It is possible to distinguish three types of luminous animals, in regard to their behavior toward illumination. Some forms (*Chætopterus*, *Ptylosarcus* (1)) when stimulated, are capable of luminescence on removal to a dark room immediately after exposure to daylight, while others (*Mnemiopsis*, *Bolina*, *Renilla* (2)) do not luminesce under these circumstances. Their power of luminescence is inhibited by daylight but is regained when kept in the dark for a short time. Still other forms (*Pelagia* (3)) show a persistent day and night rhythm of luminescence. Their luminescence is inhibited by light but if kept continually in a dark room they luminesce on stimulation only during the period corresponding to night and not during the period corresponding to day.

The behavior toward light of ctenophores, which belong in the second group, has been known for a long time. The inhibition of luminescence by sunlight was first described by Allman and has been further studied by Panceri (5), Peters (6), Harvey (1), and Moore (7). The sudden appearance of the Ctenophore, *Mnemiopsis leidyi*, at Woods Hole during the latter part of August and September gave an opportunity to test certain questions concerning the inhibition of luminescence by light.

As is well known, the luminous areas in *Mnemiopsis leidyi* are restricted to the eight rows of paddle plates (meridians) and mechanical stimulation of these anywhere will cause a luminescence at the point touched. Often a wave of luminescence will pass along the

meridian from the region stimulated. Stimulation of regions between the paddle plates (intermeridians) does not cause luminescence unless deformation of the tissue affects the paddle plate region (Moore (7)). The general nerve net work is not connected with the luminous cells and the only fact indicating that conducting pathways are involved is the propagation of the luminescence along the meridian. The propagation does not cross a cut dividing the meridian into two halves.[1]

Isolated bits of meridian tissue will give light on stimulation, but Peters (6) was never able to obtain light from a piece with less than four paddle plates. I have seen luminescence from stimulation of the tissue attached to two paddle plates. The important point is to have some of the canal present, for the luminous cells are located within the canal (Dahlgren (8)).

Small bits of canal tissue will not necessarily give light on mechanical agitation although they contain luminescent material, as can be shown by treatment with tap water or saponin, when luminescence will occur. This was demonstrated in conjunction with Professor Robert Chambers, who, with his micro dissection apparatus, dissected out a clump of canal cells and placed a glass dissecting needle in the midst of them. When this needle was moved back and forth no light appeared, but when some saponin solution was injected among them with a micro pipette, numerous luminous points appeared, similar to those observable in luminescent cœlenterates in general (1). Pieces of canal tissue of considerable length will give light if a micro needle is moved about in the tissue. It looks as if mechanical agitation causes luminescence *readily* only when a considerable mass of tissue, probably containing some nerve connections, is present.

Pieces of canal tissue with a few paddle plates attached will lose their power of luminescence in light and regain it in the dark, and the question arises, how small may fragments of ctenophores be and still show the characteristic behavior towards illumination?

[1] In some individuals propagation of a wave of light does not take place readily, but in others it does. There seems to be no doubt, however, but that a luminescence wave is really propagated along the canal, and that we are not merely dealing with a mechanical deformation of the canal tissue along its entire length.

If a dark adapted ctenophore is shaken in sea water and the sea water filtered through filter paper, an opalescent solution is obtained, which gives a few isolated points of light on shaking. When fresh water is added to it or a little saponin powder, *many* bright points of light appear, a phenomenon that may for convenience be called, "starry luminescence." The solution is opalescent and the centrifuge separates many single cells, often ciliated, and globular cell fragments, clearly seen under the microscope. The fresh water or saponin probably causes cytolysis of cells[2] containing luminous material.

This filtered extract of dark adapted *Mnemiopsis* behaves toward light as the whole animal. Its power of luminescence on adding tap water is lost after illumination and returns again in the dark, although the recovery in the dark is not complete. The amount of starry luminescence is never so great after exposure to sunlight as before.

Conversely, the extract[3] of a light adapted ctenophore, after standing in sunlight for 10 minutes, gives no luminescence when immediately brought into a dark room and tap water added, but upon standing in the dark for 10 minutes, the addition of tap water calls forth the starry luminescence.

We are therefore led to the conclusion that isolated photogenic cells (and perhaps fragments) that will pass through filter paper, have luminescent material actually decomposed by sunlight and reformed in the dark. The experiments confirm the conclusion I arrived at with *Bolina*[4] (1), that sunlight acts on the photogenic

[2] Or perhaps a dissolving of photogenic granules, granulolysis.

[3] Made by squeezing a ctenophore through cheese-cloth. Often bits of tissue with four or five paddle plates come through the cheese-cloth. After standing in the dark these luminesce readily on slight agitation but the great mass of finely divided fragments do not luminesce on agitation, although they readily luminesce on adding fresh water.

[4] A light adapted *Mnemiopsis* squeezed through cheese-cloth in the dark always gives some faint luminescence but never as great a luminescence as is obtained on treating a dark adapted animal in the same way. The light adapted *Bolina* extract will not recover its luminescent power in the dark (1) but the *Mnemiopsis* extract will. *Mnemiopsis* contains much more photogenic material than *Bolina* and this may explain the difference.

material itself, and supplement those of Moore (7) who finds that filter paper drawn over the meridians will take up material that luminesces when vigorously rubbed, or placed in isotonic KCl solution. This luminescent paper loses its power of luminescence in sunlight or artificial light but a much longer exposure is required than in the case of the whole animal. Moore interprets this to mean that *in the animal* light acts to inhibit luminescence through a nerve mechanism.

The nerve connections are strictly local, for I can confirm Moore's observation that only the region of a *Mnemiopsis* exposed to light is inhibited. The non-illuminated areas luminesce readily on stimulation and the line of demarcation between the two regions is very sharp if the beam of light used for illumination is well defined.

Light certainly acts directly on the photogenic material, even if it has in addition a more effective action through a nerve mechanism, and the question arises whether light inhibits luminescence by oxidizing the photogenic substance. One might suppose that in daylight a ctenophore luminesced and used up its available supply of material. We could not see this luminescence in the glare of day. Inhibition would thus be comparable to fatigue which occurs so readily upon repeated stimulation of the animal.[5] The question could be tested by observation of the ctenophore in a phosphoroscope, but as a phosphoroscope was not available, I have made use of ultra-violet (without the visible) light.

The spectral region which inhibits luminescence is not known with any certainty but one might predict the violet end of the spectrum to be most efficient.[6] In order to determine if the near ultra-violet has any power of inhibiting luminescence, a number of experiments

[5] It should be noted that a ctenophore shaken in a bottle for 10 minutes until only slight flashes of light appear, still has much photogenic material in it, as considerable luminescence occurs when squeezed through cheese-cloth, and the extract gives a brilliant starry luminescence in tap water. The fatigue in question is probably a nerve fatigue, not a complete exhaustion of photogenic material.

[6] A few experiments indicate that the violet end of the spectrum is much more efficacious than the red end in inhibiting luminescence. Time did not permit an accurate study of the relation.

were carried out with a quartz mercury lamp,[7] the Hanovia 110 volt A. C. Alpine Sunburner, using 720 to 740 watts after running this for some time. The animals were exposed at a distance of from 4.5 to 5.5 inches in glass dishes behind various filters which allow the near ultra-violet to pass. The far ultra-violet was of course cut out by the glass dish and filters.

If a dark adapted *Mnemiopsis*, which is brightly luminescent on stimulation, is placed in the light from the quartz mercury lamp filtered through a No. 18 Wratten ultra-violet filter, the luminescence, on stimulation at intervals, will be found to diminish gradually and in 30 minutes will have practically disappeared, even on violent stimulation. An animal in the dark, stimulated at the same intervals, will be found to be markedly luminescent at each stimulation. Hence the inhibition of luminescence is not due to fatigue but to the action of the ultra-violet light, similar in its action to sunlight. Practically no visible light passes a No. 18 filter, at least not enough to inhibit luminescence of *Mnemiopsis*, as a much greater light from a tungsten lamp in the room will not inhibit the luminescence.

Although sunlight is more effective in inhibiting the luminescence of *Mnemiopsis*, the beauty of experiments in ultra-violet light is that one can see whether the animal will luminesce during exposure, since the animal's luminescence on stimulation is perfectly apparent and very bright as compared with the slight amount of visible light which passes the No. 18 filter, or the small amount of fluorescent light from near objects. The method is therefore beautifully adapted to answer the question previously raised, whether light stimulates the animals to luminesce and uses up the available photogenic material.

Observation of the *Mnemiopsis* before the ultra-violet lamp with No. 18 filter shows that no luminescence appears, provided the animal is not disturbed. The animal is dark except for a slight bluish fluorescence along the swimming plates. If kept *undisturbed* in this ultra-violet light, no signs of luminescence appear over a

[7] I take great pleasure in thanking Dr. S. E. Pond for the use of the mercury lamp and for sharing the Nela Laboratory dark room in which many of these experiments were carried out.

period of 30 minutes, when the animal has lost completely its power of luminescence on stimulation.

Ultra-violet light does not stimulate to luminescence but a very interesting phenomenon appears when the animal is mechanically stimulated in the ultra-violet. If stimulated several times a very decided bluish luminescence *persists* after the luminescence due to stimulation has subsided. This "tonic luminescence," as it may be called, disappears when the ultra-violet is screened and reappears when the ultra-violet again strikes the animal. It behaves like a fluorescence. The animal may be stimulated in the dark and when placed in the ultra-violet the tonic luminescence is observed. It lasts for some time while the animal is in the ultra-violet light, but gradually fades out.

There are two possible explanations of the tonic luminescence. (1) It is a true chemiluminescence due to continuous or tonic stimulation of the photogenic cells in the ultra-violet light, after their luminescence has been started by mechanical stimulation. (2) The product of oxidation of photogenic material is fluorescent.

It is difficult to decide between these views. Certainly the behavior of this tonic luminescence is like a fluorescence, appearing only in the exciting radiation, but at the same time it is always associated with potentially luminescent animals. Light adapted animals (*i.e.* those exposed to sunlight) do not show tonic luminescence when stimulated before the ultra-violet. Therefore, the effect is not due to fluorescence of any non-luminescent mucus, secreted along the paddle plates on stimulation. Observation with a microscope shows that the tonic luminescence is in the canal, in the same position as the true luminescence of the animal. It seems always to be associated with once luminous material.

Animals partially fatigued by gentle shaking for 2 minutes in a bottle show tonic luminescence in the ultra-violet. Ctenophores so treated are fatigued for all practical purposes, since they give only an occasional faint flash of light. If shaken in a bottle for 15 minutes, when luminescence on stimulation is no longer possible, the tonic luminescence in ultra-violet is no longer apparent. It appears as if some material must have diffused out into the sea water when agitated for a long time. Indeed sea water in which luminescent

ctenophores have been shaken is markedly fluorescent, much more so than ordinary sea water, but fluorescence of organic material is so general that one cannot necessarily conclude that in this case we are dealing entirely with the fluorescence of photogenic substances.

On the whole, I believe the evidence points to the second explanation as correct, that the tonic luminescence is a fluorescence of the oxidation product or some intermediate substance, formed during luminescence of the animal. This view is borne out by the fact that *Chætopterus*, a luminous worm, produces a luminous slime which is very markedly fluorescent in near ultra-violet light. Moreover, the *Chætopterus* slime collected on a glass rod quite apart from the animal, is notably fluorescent, so that we are not dealing with any stimulation mechanism that may be set off in ultra-violet light. On the other hand *Cypridina* luciferin, oxyluciferin, and luciferase are not markedly fluorescent in the near ultra-violet, although I have often endeavored to observe some sign of fluorescence similar to that obtained with *Chætopterus*.

The luminous organs of the glowworm show marked fluorescence which is of a different color (more yellowish) from that of the fat-body, and of the luciferesceine, found throughout the body of the insect (see Dubois (9) and McDermott (10)). It will be of considerable interest to test other luminous animals for strong fluorescence of photogenic material.

More and more evidence is accumulating to indicate a close relation between fluorescence and chemiluminescence. Kautsky and Zocher (11) have pointed out the relations in unsaturated silicon compounds, and have shown (12) that fluorescent dyes adsorbed on non-chemiluminescent compounds (such as silico-oxalic acid) become luminescent on treatment with oxidizing agents. Evans and Dufford (13), and Dufford, Calvert, and Nightingale (14) find that many of the Grignard compounds (organomagnesium halides) are notably chemiluminescent, and their oxidation products are fluorescent in ultra-violet light. Esculin is fluorescent and most of the essential oils that are chemiluminescent in alcoholic caustic alkali, are also fluorescent.

Perhaps it is not surprising, then, to find fluorescence accompanying the brilliant photogenic material of luminous animals.

SUMMARY.

1. Small clumps of the luminous cells of *Mnemiopsis* cannot readily be stimulated mechanically but will luminesce on treatment with saponin solution. Larger groups of luminous cells (such as are connected with two paddle plates) luminesce on mechanical stimulation. This suggests that mechanical stimulation to luminesce occurs chiefly through a nerve mechanism which has been broken up in the small clumps of luminous tissue.

2. The smallest bits of luminous tissue, even cells freed from the animal by agitation, that will pass through filter paper, lose their power to luminesce in daylight and regain it (at least partially) in the dark.

3. Luminescence of the whole animal and of individual cells is suppressed by near ultra-violet light (without visible light).

4. Inhibition in ultra-violet light is not due to stimulation (by the ultra-violet light) of the animal to luminesce, thereby using up the store of photogenic material.

5. Animals stimulated mechanically several times and placed in ultra-violet light show a luminescence along the meridians in the same positions as the luminescence that appears on stimulation. This luminescence in the ultra-violet or "tonic luminescence," is not obtained with light adapted ctenophores and is interpreted to be a fluorescence of the product of oxidation of the photogenic material.

6. Marked fluorescence of the luminous organ of the glowworm (*Photuris*) and of the luminous slime of *Chætopterus* may be observed in ultra-violet but no marked fluorescence of the luminous substances of *Cypridina* is apparent.

7. Evidence is accumulating to show a close relation between fluorescent and chemiluminescent substances in animals, similar to that described for unsaturated silicon compounds and the Grignard reagents.

BIBLIOGRAPHY.

1. Harvey, E. N., *Biol. Bull.*, 1921, xli, 280.
2. Parker, G. H., *Proc. Am. Phil. Soc.*, 1920, xix, 171.
3. Heymans, C., and Moore, A. R., *J. Gen. Physiol.*, 1923–24, vi, 273.
4. Allman, G. I., *Proc. Roy. Soc. Edinburgh*, 1862, iv, 518.

5. Panceri, P., *Atti. Roy. Accad. Sc. Napoli*, 1872, v, No. 20.
6. Peters, A. W., *J. Exp. Zool.*, 1905, ii, 103.
7. Moore, A. R., *J. Gen. Physiol.*, 1923–24, vi, 403.
8. Dahlgren, U., *J. Franklin Inst.*, 1916, clxxxi, 243.
9. Dubois, R., *Bull. Soc. Zool. France*, 1886, xi, 1.
10. McDermott, F. A., *J. Am. Chem. Soc.*, 1911, xxxiii, 410.
11. Kautsky, H., and Zocher, H., *Z. Physik*, 1922, ix, 267.
12. Zocher, H., and Kautsky, H., *Naturwissenschaften*, 1923, xi, 194.
13. Evans, W. V., and Dufford, R. T., *J. Am. Chem. Soc.*, 1923, xlv, 278.
14. Dufford, R. T., Calvert, S., and Nightingale, D., *J. Am. Chem. Soc.*, 1923, lxv, 2058.

THE NUMBER OF BLOOD CORPUSCLES IN FISHES IN RELATION TO STARVATION AND SEASONAL CYCLES.

By ARTHUR A. SCHAEFER.

(From the Zoological Laboratory, University of Wisconsin, Madison.)

(Accepted for publication, October 31, 1924.)

In poikilothermal animals metabolic activity is known to be much less during periods of hibernation, but the relation of the blood to such changes has been very little studied. Heesen[1] investigated the blood of amphibians and found that leucocytes were at a minimum in February and reached a maximum in April, during the breeding season. Though the erythrocytes were more variable, they also occurred in greater numbers during the spring.

In October, 1923, the writer brought forty pumpkinseeds, *Eupomotis gibbosus* L. Cuvier and Valenciennes, from Lake Mendota into the laboratory. These were kept in a large glass aquarium through which a slow stream of filtered, well aerated water ran continually. The temperature varied little each day, but throughout the course of the experiments ranged between 12° and 18°C, average being about 14°C. During the first few days fecal strings were removed at intervals from the bottom of the aquarium, but after 2 weeks no more appeared. Only one fish died, on May 14, 1924, and all others appeared to remain in good condition.

Various methods of obtaining blood for counts were tried, but the one finally used was that of cutting off the tail and drawing blood from the aorta. Because the erythrocytes of fishes are nucleated, it was not found practical to make differential counts by the methods commonly used for human blood. The results obtained, therefore, relate to counts of leucocytes and erythrocytes combined. A Thoma-Zeiss chamber was used and an entire square millimeter was counted in each case. Toison's fluid was used to dilute the blood 100 times, and gentian violet served as a stain.

[1] Heesen, W., *Z. vergl. Physiol.*, 1924, i, 500.

On October 11, 1923, counts were made of blood from six fishes which gave the following results per cubic millimeter: (1) 2,540,600; (2) 2,509,300; (3) 2,295,000; (4) 2,400,000; (5) 2,382,400; (6) 2,465,000.

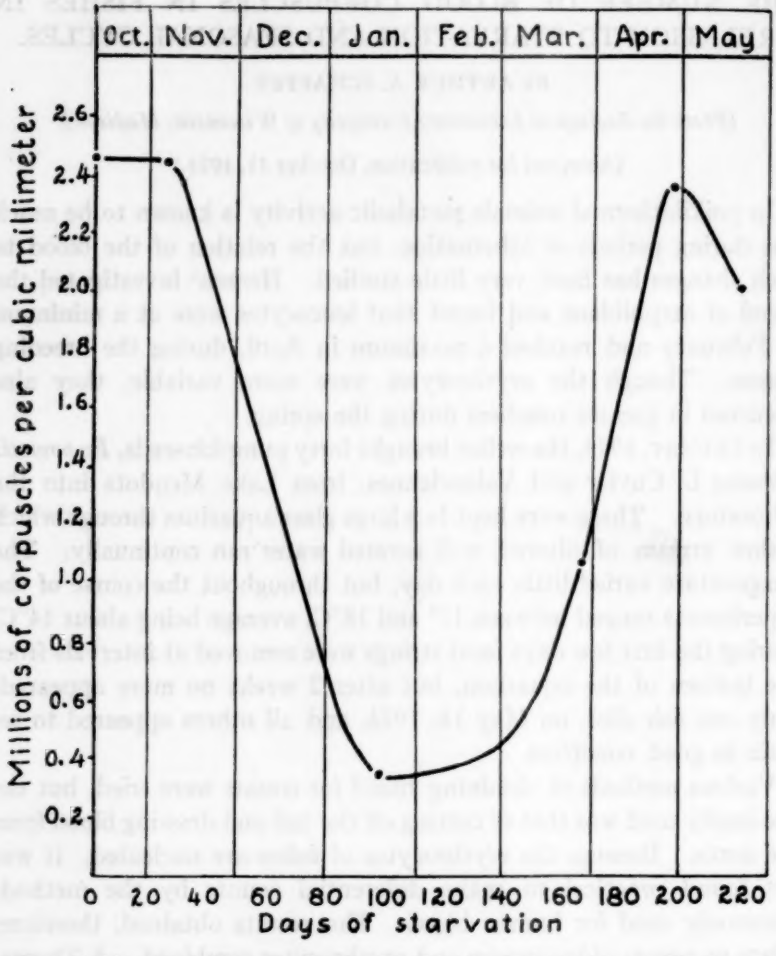


FIG. 1. Number of corpuscles in the blood of starved pumpkinseeds from October 11, 1923 to May 16, 1924.

After the remaining fishes had been starved 27 days (November 8, 1923) the blood samples from four were counted: (1) 2,493,700; (2) 2,375,000; (3) 2,256,200; (4) 2,537,500. On the 97th day of starvation

(January 17, 1924) two fishes gave the following counts: (1) 338,000; (2) 327,000, about 85 per cent decrease. After 168 days (March 28, 1924) there was a marked increase but the numbers were far below those observed in October: (1) 1,106,200; (2) 1,012,500; (3) 1,100,000; (4) 1,087,500. After 196 days of starvation (April 25, 1924) there was a further increase and the count practically returned to the average for October: (1) 2,256,000; (2) 2,455,000; (3) 2,295,000; (4) 1,087,500. After 217 days (May 16, 1924) the counts were 17 per cent below those in October: (1) 2,175,000; (2) 1,875,000.

The fishes were all weighed at the beginning of the experiments and each was weighed again just before its blood was examined. There was a regular decrease in weight throughout the period of observation: after 27 days, 2.7 per cent; after 97, 21.8; after 196, 30.6; after 217, 38.0. The blood counts were not related to the change in weight for they went down during the winter and came back to practically normal in the spring (Fig. 1). The decrease in the number of corpuscles at the end of the experiment was perhaps on account of starvation.

The observations indicate that fishes develop anemia during hibernation under the conditions of the experiment and that the corpuscle count may return to normal in the spring even when food is lacking.

NOTE ON THE EXCITATION AND INHIBITION OF LUMINESCENCE IN BERŒ.*

BY C. HEYMANS AND A. R. MOORE.

(From the Pharmacological Laboratory of Ghent University, Ghent, Belgium, and the Physiological Laboratory of Rutgers University, New Brunswick.)

(Accepted for publication, November 3, 1924.)

Berœ ovata is a large ctenophore which, when dark adapted, can display a brilliant blue-green luminescence. The luminous organs are distributed along the meridians marked by the swimming plates. In a dark adapted animal, weak stimulation causes local luminescence while strong excitation calls forth general luminescence from the meridians of the entire animal.[1] The rapidity of the conduction in general luminescence suggests that the mechanism is nervous.

In nature the phenomena of luminescence occur only at night. If specimens of *Berœ* be taken from the light at any time of day and placed in the dark room they at first show no luminescence even after vigorous excitation. But after half an hour in the dark they exhibit general luminescence when touched or agitated.

In order to determine the effect of ions on the luminescence of *Berœ*, experiments were carried out similar to those which we have reported for *Pelagia*.[1] In van't Hoff's solution *Berœ* behaves with reference to luminescence just as in sea water. If, however, one of the cations be omitted from the solution, the luminescent reactions of *Berœ* are greatly modified.

* The experiments on the reactions of *Berœ* and its luminescent material with ions were done at Naples in the spring of 1923. The quantitative data for the relation between the intensity of light and time of exposure required for the inhibition of luminescence were the result of experiments on specimens of *Berœ* taken from Barnegat Bay, New Jersey, October, 1924.

[1] Heymans, C., and Moore, A. R., *Compt. rend. Soc. biol.*, 1923, lxxxix, 430; *J. Gen. Physiol.*, 1923–24, vi, 273.

In van't Hoff's solution minus calcium, general luminescence is inhibited after 5 minutes, so that strong mechanical excitation of the animal yields only local luminescence. This proves the necessity of calcium ions for nervous conduction of the impulse causing general luminescence. Similar results are obtained if potassium alone is omitted from the van't Hoff solution.

The omission of magnesium from van't Hoff's solution leads to effects quite the reverse. When placed in such a solution *Berœ* responds by spontaneous and almost continuous luminescence, showing a condition of hyperirritability induced by the presence of Ca and K ions unantagonized by the Mg ion. These facts prove that the appearance and intensity of luminescence, as they result from nervous conduction, are dependent upon the composition of the external medium.

Luminescent indicator paper, prepared by spreading the luminescent material on filter paper, does not show luminescence if kept in the air, in sea water, in NaCl, or $MgCl_2$ or saccharose solutions. The solutions used are in all cases isosmotic with sea water and of reaction pH 7.8. In a solution of $CaCl_2$ the paper shows luminescence and remains glowing for about 12 minutes. $SrCl_2$ yields a similar result. In $BaCl_2$ the glow is briefer. In KCl solution the glow lasts 2 minutes; in K_2SO_4 solution, about the same length of time. The addition of alkali to the salt solution increases the intensity of the luminescence and at the same time shortens the duration of the glow. For example, when a piece of luminescent paper was put into a solution of 20 cc. $CaCl_2$ + 0.1 cc. N NaOH, a very bright luminescence appeared which lasted $2\frac{1}{2}$ minutes. These results extend the observations made with the luminous substance of *Pelagia* and confirm our former conclusions.

Just as in the case of *Pelagia* and *Mnemiopsis*,[2] so in *Berœ* exposure to light causes inhibition of luminescence. In order to determine the exact relations between the quantity of light and the time of exposure necessary for the suppression of luminescence in *Berœ* the following series of experiments was carried out.

The method was the same as that employed in the experiments with *Mnemiopsis*,[2] except that the light used was of 670 c.p. The tem-

[2] Moore, A. R., *J. Gen. Physiol.*, 1923–24, vi, 403.

TABLE I.

Relation between Intensity of Light and Time of Exposure Required for the Inhibition of Luminescence.

Intensity.	No. of animals.	Time.	$K = I \times t$
meter candles		*min.*	
1863	9	30	55,890
2680	12	22	58,960
4188	12	13	54,444
7437	12	8	59,496
		Weighted average = 57,285	

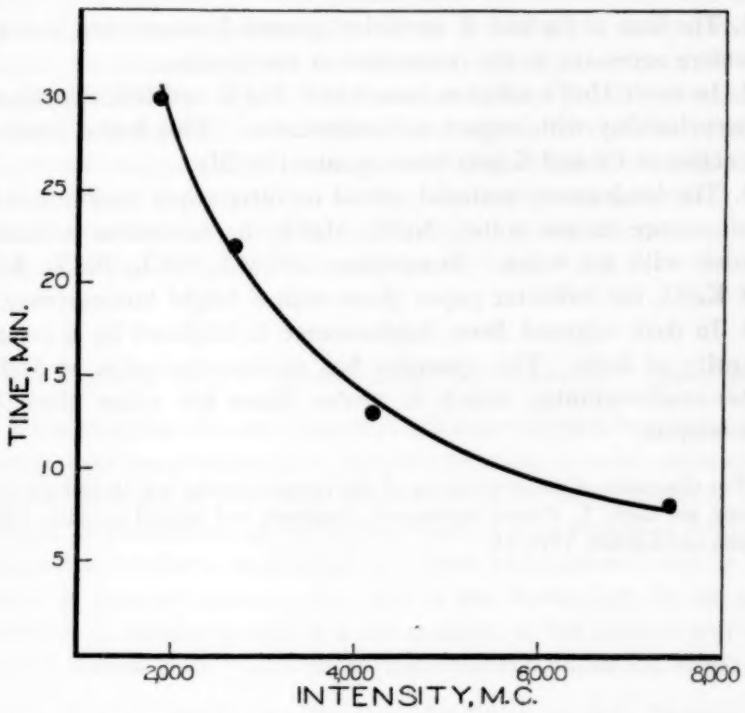


FIG. 1. Abscissæ represent intensities of light (I) and ordinates the average time (t) required at the intensity given to inhibit luminescence. The curve represents the calculated values of t for the intensities used, when $K = 57,285$ in which $K = I \times t$.

perature was 18–20°C. Four intensities were used with the result shown in the table and figure. The method of making the observations and computing averages was the same as described in the work on *Mnemiopsis*.

The product of the intensity of the light by the time necessary for suppression of luminescence in *Berœ* is a constant. This proves that *Berœ* is affected by light according to the Bunsen-Roscoe photochemical law.[3] The constant for *Berœ* has an average value of 57,285 meter-candle-minutes, which is approximately twelve times the magnitude of the corresponding constant obtained for *Mnemiopsis*.

CONCLUSIONS.

1. The ions of Ca and K condition general luminescence, and are therefore necessary to the conduction of the impulse.

2. In van't Hoff's solution from which Mg is omitted, *Berœ* shows hyperirritability with respect to luminescence. This is the result of the action of Ca and K ions unantagonized by Mg.

3. The luminescent material spread on filter paper does not show luminescence in sea water, NaCl, $MgCl_2$, or saccharose solutions isotonic with sea water. In solutions of $CaCl_2$, $SrCl_2$, $BaCl_2$, KCl, and K_2SO_4 the indicator paper glows with a bright luminescence.

4. In dark adapted *Berœ*, luminescence is inhibited by a certain quantity of light. This quantity has an average value of 57,285 meter-candle-minutes, which is twelve times the value given by *Mnemiopsis*.

[3] For discussion of the application of the Bunsen-Roscoe law to biological reactions, see Loeb, J., Forced movements, tropisms, and animal conduct, Philadelphia and London, 1918, 83.

CONDUCTIVITY AS A MEASURE OF THE PERMEABILITY OF SUSPENDED CELLS.

By S. C. BROOKS.

(From the Division of Pharmacology, Hygienic Laboratory, United States Public Health Service, Washington, D. C.)

(Accepted for publication, November 10, 1924.)

The electrolytic conductance of living cells has been used extensively as a measure of their permeability to electrolytes, and more particularly of the permeability of an hypothetical semipermeable plasma membrane supposed to delimit the protoplasm from the surrounding medium. There are, however, many possible sources of error in making such an interpretation, especially when suspensions of cells are studied. This paper is a critical consideration of this phase of the subject, and exposition of the precautions and corrections suggested by rather extended intensive study of the conductance of bacteria, red blood cells, unicellular algæ, and yeast.

I.

The Influence of Cell Volume on the Conductance of Suspensions.

When studying the conductance of cell suspensions it becomes necessary to take many precautions in order to arrive at a fair estimate of the conductance of the cells themselves. The importance of concurrent determinations of the conductance of the suspending fluid and suspension has been emphasized by Green and Larson[1,2] and by the writer in previous papers[3,4] in which it was shown that the net conductance as therein defined is a fair measure of the relative conductance of normal cells. But under abnormal conditions the volume of

[1] Green, R. G., *Papers from The Mayo Foundation for Med. Education and Research and the Med. School*, 1921, i, 577.

[2] Green, R. G., and Larson, W. P., *J. Infect. Dis.*, 1922, xxx, 550.

[3] Brooks, S. C., *Proc. Soc. Exp. Biol. and Med.*, 1921–22, xix, 284.

[4] Brooks, S. C., *J. Gen. Physiol.*, 1922–23, v, 365.

the suspended cells may change, and the net conductance (which is in reality a measure of resistance) must be corrected for the volume of the cells if their true relative conductance is to be determined. How is this volume to be estimated? This can be answered, at least in part, for red blood cells and bacteria, which will be considered separately. Adequate study has not yet been given to the cases of yeast or the green alga *Chlorella*.

a. Red Blood Cells.—The true volume of red blood cells has been sought by many methods which fall under about five heads: quantitative determination of certain ingredients in defibrinated blood, serum, and serum-free cells; estimation of certain substances in cells and surrounding fluid before and after dilution; estimation of the concentration in the surrounding fluid of a substance added to a suspension of blood cells; relative conductance of suspension and suspending fluid; and centrifugation of a cell suspension in a graduated tube until the mass of cells becomes translucent (hematocrit). In the hematocrit method the cell mass is supposed to become translucent only when all differences in refractive index at the fluid-cell surfaces, are eliminated by the elimination of all the suspending fluid. In the conductivity method the cells are assumed to be non-conductive, the observed slight conductivity of the densest obtainable masses of cells being attributed to the presence of residual traces of suspending fluid. The first three methods depend upon the assumption that the substance estimated is not taken up or given off by the cells.

We propose to show that of these five methods the hematocrit is the most nearly correct. The cell volume estimated by this method is consistently somewhat above that calculated from the other methods, and it has been with some reason that the hematocrit method has been distrusted. The possible errors in any of the other methods will be shown to be consistently in favor of minimizing the volume of the cells, and evidence will be presented to show that the conductivity data have not been correctly interpreted.

The first method was used by Bunge[5] who determined the sodium content of defibrinated blood and of its serum; this method is available

[5] von Bunge, G., Lehrbuch der Physiologischen und Pathologischen Chemie in Neun und Zwanzig Vorlesungen für Ärtzte und Studirende, Leipsic, 4th edition, 1898, 228.

only for blood whose cells contain no sodium, as is assumed to be the case for the pig, horse, or rabbit. Hoppe-Seyler's method[6] can best be illustrated by data taken from an experiment by Stewart.[7] The protein and hemoglobin content of 100 gm. of the defibrinated blood was 17.360 gm.; of 100 gm. of *washed* corpuscles, 14.254 gm.; the protein content of 100 gm. of serum was 5.424 gm. The serum in 100 gm. of defibrinated blood is given by the calculation

$$\frac{17.360 - 14.254}{5.424} \times 100 = 57.26 \text{ gm.}$$

But if 2.2 per cent of the protein and hemoglobin of the cells had been lost during washing, the figure 14.254 should have been 14.575, and the relative amount of serum should have been more than 10 per cent less, *i.e.* 51.35. A very small loss of protein and hemoglobin during washing, even one so small as to pass unnoticed, will greatly decrease the apparent volume of cells in a given sample of blood.

The second method was suggested by Bleibtreu[8] who calculated the original volume of serum in defibrinated blood by estimating the protein nitrogen before and after dilution with 0.6 per cent NaCl. This presupposes that no change of cell volume occurs, and that no protein nitrogen escapes from the cells under the new conditions set up by dilution. The method has been severely criticized on account of both of these errors by Hamburger,[9] Biernacki,[10] Eykman,[11] and Hedin[12] and has been shown not to give self-consistent results when different degrees of dilution are compared. Scott[13] has shown that both protein and non-protein nitrogen are lost by red blood cells to Ringer solution in which they are being washed. The change of cell volume might be made relatively negligible by using a strictly isotonic diluent, but if protein nitrogen diffused from the cells the decrease in

[6] Hoppe-Seyler, F., Handbuch der physiologisch-und pathologisch-Chemischen Analyse, Berlin, 6th edition, 1893, 274.

[7] Stewart, G. N., *J. Physiol.*, 1899, xxiv, 356.

[8] Bleibtreu, M., and Bleibtreu, L., *Arch. ges. Physiol.*, 1891–92, li, 151.

[9] Hamburger, H. J., *Zentr. Physiol.*, 1893–94, vii, 161.

[10] Biernacki, E., *Z. physiol. Chem.*, 1894, xix, 179.

[11] Eykman, C., *Arch. ges. Physiol.*, 1895, lx, 340.

[12] Hedin, S. G., *Arch. ges. Physiol.*, 1895, lx, 360.

[13] Scott, F. H., *J. Physiol.*, 1915–16, l, 128.

concentration of protein nitrogen as a result of dilution would be too small. This would in turn lead to the conclusion that the original volume of serum was greater than it actually was, and in consequence that the cell volume was less than it really was. The liberation from the cells of a very minute amount of protein nitrogen would account for the difference between this and the hematocrit method. Similar comment may be made on other dilution methods and hence absolute certainty as to cell volume cannot be attained by these methods.

The addition of substances to serum is also unreliable. When introduced by Stewart,[7] who added hemoglobin to defibrinated blood, there seemed to be every reason for supposing the erythrocytes to be impermeable to hemoglobin and hence to be incapable of taking any hemoglobin out of the solution. But the recent work of Brinkman and von Szent-Györgyi[14] on the reversion of hemolysis, as well as some previous work mentioned by them make it entirely probable that some of the hemoglobin which Stewart introduced into the defibrinated blood was taken up in some way by the cells.

The work of Bayliss,[15] while casting considerable doubt on the main conclusion of Brinkman and von Szent-Györgyi, does not disprove the supposition that at least some of the hemoglobin is absorbed by red blood cells under the conditions described. Such an absorption of hemoglobin, occurring in Stewart's experiments, would reduce the observed concentration of hemoglobin in the serum, and lead to an overestimate of the volume of serum, and to the calculated volume of erythrocytes being again too low. All three analytical methods probably lead to an underestimate of the volume of cells in a given suspension of erythrocytes.

The conductivity method also was introduced by Stewart[7] as a method of determining the volume of erythrocytes in defibrinated blood. Formulæ developed empirically by Stewart,[7] Róth,[16] and Oker-Blom[17] are theoretically unsound, since they give impossible values for the conductance of cells alone or suspending fluid alone. Formulæ were developed by the writer which gave the theoretical limits between

[14] Brinkman, R., and von Szent-Györgyi, A., *J. Physiol.*, 1923–24, lviii, 204.
[15] Bayliss, L. E., *J. Physiol.*, 1924, lix, 48.
[16] Róth, W., *Zentr. Physiol.*, 1897–98, xi, 271.
[17] Oker-Blom, M., *Arch. ges. Physiol.*, 1900, lxxix, 510.

which the conductance of a suspension should lie, but did not lead to a method of calculating the resistance exactly.[18]

Formulæ recently published by Fricke[19,20] are much more satisfactory. In these formulæ there appears the constant β, a function of the shape and conductance of the internal phase, here the suspended cells, and of a quantity λ whose significance is not given. In Fig. 1 of the latter paper,[20] β appears as a function of $\frac{a}{b}$, (a and b being the axes of spheroidal particles), and of $\frac{k_2}{k_1}$ in which k_2 and k_1 are the conductivities of the suspended and suspending media, respectively. In applying the formulæ to the conductivity of suspensions of erythrocytes as observed by Stewart[7] Fricke obtains good agreement between observed and calculated volumes by assuming the cells to be nonconductive and the ratio of diameter to thickness to be as 4:1. This gives $\beta = -1.91$. However, the red blood cells are not exactly spheroidal, but rather biconcave; and furthermore the ratio of their axes is still very much in doubt; and, as Ponder[21] so ably points out, they will be in doubt until methods are available much more precise than any we now possess. Apparently the ratio is more than 4:1

[18] Paper presented at the meetings of the American Physiological Society, Toronto, Dec. 27, 1922. Since these formulæ have not heretofore been presented in print they are given here as a matter of record. If G is the specific conductance of the suspension as a whole, g that of the outer phase, *e.g.* suspending fluid, kg that of the inner phase, *e.g.* cells, and n the relative volume of cells in parts per mille of the suspension, then G lies between

$$g\left[1 - \frac{n(1-k)}{(n - 10n^{2/3})(1-k) + 1{,}000}\right]$$

and

$$g\left[1 - \frac{n(1-k)}{100n^{1/3}(1-k) + 1{,}000k}\right]$$

In some cases k is so small that the first equation gives impossible values, showing that the current paths assumed in developing this equation are not those actually followed in these cases.

[19] Fricke, H., *J. Gen. Physiol.*, 1923–24, vi, 375.

[20] Fricke, H., *J. Gen. Physiol.*, 1923–24, vi, 741.

[21] Ponder, E., *Quart. J. Exp. Physiol.*, 1924, xiv, 37.

rather than less.[22] The rather doubtful value of this ratio gives to the constant β quite a range of possible values at any given value of $\frac{k_2}{k_1}$.

Therefore when Fricke[20] assumes that red blood cells act as non-conductors $\left(\frac{k_2}{k_1} = 0\right)$, and applies his formulæ to the data given by Stewart[7] and obtains good agreement, it does not follow that the premises are correct. If Stewart's data on the cell volume, obtained by the analytical method, are too low, then better agreement may be obtained by assuming $\frac{k_2}{k_1} > 0$ and β correspondingly smaller. Such a calculation has been carried out for $\frac{k_2}{k_1} = 0.05$ and $\beta = -1.74$; this value of β corresponds to a ratio of diameter to thickness of the assumed spheroids equal to 4.2 : 1 approximately.[23]

[22] The figures given by different writers vary widely: Starling (Starling, E. H., Principles of human physiology, Philadelphia, 2nd edition, 1915, p. 811) gives the diameter of human erythrocytes as 7.1–7.8μ, thickness at edge 2.5μ, at centre 1–1.5μ; this gives a mean ratio very nearly 4:1. Ponder (Ponder, E., *Proc. Roy. Soc. London, Series B*, 1922–23, xciv, 102) gives the corresponding dimensions as 7.8, 2, and 1.5μ, respectively; ratio 4.5:1; but as a result of recalculation from the data of Gulliver (Gulliver, G., *Proc. Zool. Soc. London*, 1875, 474). Comes to the conclusion that the ratio for mammalian cells is about 3:1 at the edge and 4:1 at the center. Since Gulliver's data were obtained by measurements of cells in dried smears (Gulliver, G., *Phil. Mag.*, 1840, xvi, series 3, 23) it seems to the writer to be rather dangerous to transfer these data to cells lying in defibrinated blood. Schenck and Gürber (Schenck, F., and Gürber, A., Outlines of human physiology, translation, W. Zoethout, New York, 1900, p. 53) give the average diameter and thickness of human red blood cells as 7–8μ and 1.6μ, respectively; the ratio is 4.4–5.0:1.0. These, which are fairly representative figures, suggest a figure somewhat above 4:1 as the most probable value of the mean ratio of diameter to thickness in the case of the cells under consideration.

[23] This value seems to be about that to be expected by extrapolation along the $\frac{a}{b}$ axis for $\frac{k_2}{k_1} = 0.05$ in Fricke,[20] Fig. 1. Formulæ published since this paper was written (Fricke, H., *Phys. Rev.*, 1924, xxiv, 575) make possible the exact calculation of β, but this would not materially affect the result of my recalculation.

The agreement obtained in this way is better than that given by Fricke, as may be seen in Table I.

TABLE I.

Comparison of the volume of erythrocytes in 100 volumes of suspension as observed and as calculated by the use of the formula

$$\frac{k-k_1}{k-k_2}\left(1-\frac{k_2}{k_1}\right)=\beta\frac{\rho}{1-\rho}$$

in which ρ = concentration per cent, k, k_1, and k_2 the conductivities of the suspension, suspending fluid, and cells, respectively, and β a constant. In the recalculation ρ is corrected by increasing ρ observed by a uniform proportion.

Calculation by Fricke.			Recalculation.		
ρ observed	ρ calculated.	Error.	ρ corrected.	ρ calculated.	Error.
		per cent			*per cent*
90.7	88.4	−2.5	99.9	97.4	−2.5
82.1	81.9	−1.5	90.4	89.5	−0.9
74.5	74.4	−0.1	82.0	82.1	0.1
67.8	68.0	0.2	74.75	74.7	0.05
61.6	62.1	0.8	67.9	68.4	0.7
56.1	56.8	1.2	61.8	62.3	0.7
51.0	51.9	1.8	56.2	57.2	1.7
46.4	47.4	2.1	51.1	52.2	2.1
42.2	42.9	1.7	46.2	47.2	2.1
41.0	41.3	0.7	45.2	45.4	0.3
38.4	39.0	1.4	42.3	42.9	1.6
31.9	32.0	0.2	35.2	35.2	0.0
26.4	26.7	1.1	29.1	29.4	1.2
21.8	22.0	0.9	24.0	24.1	0.4
18.1	18.3	1.1	19.95	20.0	0.25
15.3	15.2	−0.7	16.87	16.65	−1.3
11.4	10.8	−5.3	12.55	11.88	−5.4
Totals.....	—	23.8	—	—	21.3
Mean error.....		1.37	Mean error.....		1.25
" root square error.....		1.81	" root square error....		1.79

The average per cent discrepancies by Fricke's and the writer's calculations are 1.37 and 1.25, and the root mean square discrepancies, 1.81 and 1.79, respectively. Either standard of comparison favors the theory that the cells are slightly conductive and that Stewart's method of estimating their volume gave results which were too low.

Fricke's formulæ have been applied to some of the writer's data, with the results given in Table II. Assuming that the cells retain their natural shape and that this is approximately that of an oblate spheroid the ratio of whose axes $\frac{a}{b} = \frac{1}{5}$, values were assumed for the

TABLE II.

Analysis of Data on the Conductance of Various Suspensions of Red Blood Cells as a Function of Their Volume Concentrations.

Suspension.	α	$\alpha\|_\infty$	$\frac{\alpha}{\alpha_\infty}$	ρ calculated.	ρ observed.	If $k_2 = 0$ and ρ is correct.	
						β	$\frac{a}{b}$
Horse cells in plasma (whole blood).	−1.91	0.20	0.05	0.500	0.500	−1.74	0.32
" " " serum (defibrinated blood).	−1.79	0.20	0.09	0.514	0.516	−1.49	1.00 (?)
Horse, washed cells in balanced solution.	−1.30	0.20	0.21	0.482	0.482	−0.99	Impossible.
Rabbit, washed cells in balanced solution.	−1.48	0.20	0.15	0.263	0.263	−1.33	"

The symbols have the same significance as in Table I.

conductivity of the cells $\left(\frac{k_2}{k_1}\right)$, corresponding values of the constant β deduced from Fricke's Fig. 1,[20] and the volume concentration ρ calculated therefrom. Values of $\frac{k_2}{k_1}$ and β were thus found which led to values of ρ very close to those observed. These are the values given in Table II.

In the last two columns are given the values which must be assumed for β and $\frac{a}{b}$ if the cells are assumed to be non-conductive. The assumption is seen to lead to rather strange or impossible conclusions.

Change of shape probably compensates for part of the observed differences in the ratio $\frac{k_2}{k_1}$, but these values fit the facts much better than the value $\frac{k_2}{k_1} = 0$ assumed by Stewart, Fricke, and others.

One great service of Fricke is that he has shown, theoretically at least, the great influence which the shape of suspended particles exerts on the conductance of their suspensions. Ponder has shown[24] that erythrocytes in swelling undergo great change of shape, and it follows that no study of the conductance of red blood cells is of any value unless swelling or shrinkage of the cells is shown to be absent, or properly taken into account.

All the methods of estimating cell volume have been shown to rest upon more or less dubious assumptions. To the writer the assumption underlying the hematocrit method, namely that translucence of the sediment indicates freedom from serum, seems to be the most objective, and the least dubious of all. This is also the conclusion reached by Ege[25] who has already called attention to many of the points noted in the present paper.

It is, therefore, evident that we are still at liberty to assume that red blood cells are conductive, which seems to be almost unavoidable when one considers their great permeability to anions and hydrions.[26] Further, one may assume that the hematocrit or an equivalent centrifugation method gives the most nearly correct values of the volume of red blood cells present in a suspension.

In practice, a centrifugation of 5 minutes with a precipitating force at the bottom of the 15 cc. centrifuge tube equal to about 1,600 × gravity was usually enough to bring the volume of the cells to within about 1 per cent of the volume when the cells were centrifuged to translucence. Some procedures, such as heating, so reduce the specific gravity of the cells that this method of determining the volume is useless. Under most conditions, erythrocyte volume can be, and has in my work been determined by centrifugation.

[24] Ponder, E., *Proc. Roy. Soc. London, Series B*, 1922–23, 102.

[25] Ege, R., *Biochem. Z.*, 1920, cix, 241.

[26] For the literature see Höber, R., Physikalische Chemie der Zelle und der Gewebe, Leipsic, 5th edition, 1922, pp. 443–54.

b. Bacteria.—When we study bacteria, however, we find that sedimentation depends so much upon the surface charge of the cells that centrifugation is less satisfactory than for erythrocytes. Apparent changes in volume may be due to changes in the reaction of the medium, or to the addition of some new substance,[27] and all endeavors to determine the actual conductivity of bacteria must be made under such conditions as to keep their surface potential constant. This must be considered in addition to the factors mentioned in the discussion of erythrocyte volume, factors, which in the case of bacteria, are of much less relative importance. Certain bacteria have more than one point of no potential, *e.g. Bacillus cereus*, at pH 3.0 and 10.0[28] but in any event these reactions represent highly unfavorable conditions under which the cells cannot long survive. In the case of some bacteria, however, a pH can be chosen and maintained at which the potential of the cells is low enough to allow fairly efficient centrifugation, and yet have no serious effects on the viability of the bacteria during the course of the experiment.

The absolute volume of bacterial cells is inaccessible to determination during ordinary experiments, but with proper precautions, the relative volume, and hence, to a limited extent, the changes of electrical conductivity, can be followed by the writer's method of centrifugation and resuspension.

II.

The Viability of Bacteria in Suspensions.

In experiments to determine the effect of reagents on the electrolytic conductance of living cells it is important to know that the cells are really living. This is simple in the case of red blood cells which may be considered to be living (as much as any red blood cell may be considered alive) unless or until it is hemolyzed.

Bacteria may be plated on suitable media and the number of colonies compared with the number of organisms present in a stained smear. Using known volumes and dilutions it is to be expected that clumping of bacteria would make the number of colonies growing on the plates

[27] Eggerth, A. H., and Bellows, M., *J. Gen. Physiol.*, 1921–22, iv, 669.

[28] Winslow, C.-E. A., Falk, I. S., and Caulfield, M. F., *J. Gen. Physiol.*, 1923–24, vi, 177.

less than that expected by calculation from the number of stained organisms countable in the smears. This was found to be the case when four independent determinations were made in the case of a suspension of *Escherichia communior*: (*Bacillus coli communior*, fg strain):[29] the number of colonies was 52.0 ± 1.2 per cent of the calculated number.[30]

Apparently a large proportion of the bacteria were viable; the possible effects of any dead bacteria present can only be guessed at, since the manner of killing affects the subsequent characteristics of the cells.[4]

III.

Interpretation of Data during Progressive Change in the Conductance of Cells and Surrounding Fluid.

Still another difficulty is often encountered in studying the conductance of suspended cells: the conductance of both cells and suspending fluid may be undergoing rapid change. It is misleading to determine the net conductance from determinations of the resistances of the suspending fluid and suspension when they are separated by an interval of several minutes during which changes are occurring in both. Apparently the best approximations are to be made by assuming that the conductance found for the suspending fluid is that proper to some time during centrifugation, presumably when about half the cells are already gathered into a sediment. Plotting the observed resistances at these times, and the resistances of the suspension at the moment of observation, the most probable values of the net conductance can be determined by interpolation.

[29] Rogers, L. A., Clark, W. M., and Davis, B. J., *J. Infect. Dis.*, 1914, xiv, 411. *Cf.* Wilson, J. A., and Vollmar, C. J., *Ind. and Eng. Chem.*, 1924, xvi, 367.

[30] The suspension contained cells grown on pea extract trypsin agar; slants were inoculated from stock cultures on nutrient agar, grown 24 hours at 37.5°C., the growth of each tube then transferred to fresh medium in Kolle flasks and grown 18 to 20 hours more at 37.5°C. The growth was then taken up in unbuffered Ringer-Locke solution, washed several times with the same, and finally suspended in about four times its volume of the same solution. The pH of the suspension was 8.8 to 9.0, varying slightly on different days.

Experience has shown that these interpolations usually follow with very little uncertainty. The permissible range of variation in the placement of the smoothed curves is so limited that any error involved is obviously smaller than other experimental errors. Such interpolations as these will be considered in a forthcoming publication.

IV.

The Interpretation of Conductance Data in Terms of Permeability.

Let us finally examine what is meant by the term permeability of a membrane. By derivation and in its physicochemical sense it may be defined as the freedom with which the membrane allows a substance or substances to pass through it; the membrane may be thought of as hindering in some way the free diffusion of ions; the greater the hindrance the less the permeability. It would seem possible to measure permeability by the rate at which the substance in question passes through the membrane relative to the rate of free diffusion in the absence of a membrane. If permeability remained constant, then doubling the concentration gradient should double the rate of diffusion both with and without the presence of the membrane, but it should leave the ratio between the two unchanged. In general, however, changes in the constitution of the solutions bathing a membrane produce changes in its permeability; this is particularly true of living cells, whether their semipermeability be considered to be due to the presence of a plasma membrane, or be referred to the protoplasm as a whole, considered as a membrane.

Practically, the permeability of a cell is that property which determines how rapidly, if at all, substances pass into and out of the cell. The definition given above is the only one which is satisfactory from this point of view, and the word permeability will be so understood in this and the following papers by the writer.

When this definition is applied to the use of conductance as a measure of permeability it is apparent that changes in conductance of the cell or any part of it in which its semipermeability is supposed to reside, will change the conductance of a mass or suspension of cells whether or not the permeability as just defined undergoes any change. Electrolytic conductance measures two independent quantities—per-

meability and conductivity, the latter being thought of as a function of the dissociation constants, ionic mobility, and concentration of the electrolytes present. It will, of course, be recognized that the semipermeable region may owe some of its semipermeability to an effect which it produces on the mobility, dissociation, or other characteristics of an electrolyte, but these can also change independently of the semipermeable structure or phase. Therefore, electrolytic conductance cannot be used as a measure of permeability unless such independent changes in conductivity can be excluded.

The writer has shown that such changes probably occur [3,4,31] and has called attention to the misleading conclusions as to permeability which might have been drawn from the data presented.

The conclusion that electrolytic conductance does not necessarily measure permeability has been stated independently by Robbins.[32] [33]

The number of pitfalls in the interpretation of the measurements of electrical conductance of cell suspensions is impressive. It is not to be wondered at that so little of permanent value has been achieved by such studies. We may take as one last example the preliminary report by McClendon[34] on the conductance at different frequencies of a sediment of erythrocytes "containing a small percentage of serum." The observed specific conductance at 10^3 ∞ was 0.0010 ohms^{-1}, and at 10^6 ∞ it was 0.0014 ohms^{-1}, an increase of 40 per cent, and one which is apparently very striking. Suppose, however, that the volume of the erythrocytes had changed by less than 5 per cent, *e.g.* from 95.0 per cent to 90.5 per cent of the total volume of "sediment," then, without change in any other factor such as shape or conductivity of cells or serum, this slight change alone would account entirely for the observed difference in conductance. While it may justly be claimed that there is no *a priori* reason for supposing any such change to occur, the fact remains that the data as presented are not adequate proof that there is any difference in the conductance of the erythrocytes at the two different frequencies.

[31] Brooks, S. C., *Am. Assn. Advancement Sc.*, Boston, Dec. 29, 1922; *XI. Internat. Physiol. Cong.*, Edinburgh, July 25, 1923; *Brit. Assn. Advancement Sc.*, Liverpool, Sept. 14, 1923; and papers in course of publication.

[32] Robbins, W. J., *Am. Assn. Advancement Sc.*, Toronto, 1921 (discussion).

[33] Robbins, W. J., *Am. J. Bot.*, 1923, x, 412.

[34] McClendon, J. F., *Science*, 1924, lx, 204.

Suspended cells are living things in a state of labile equilibrium with their environments; and no attempt at physical analysis of their electrolytic behavior, no matter how excellent it may be as an analysis, can interpret living cells if they are treated as inanimate, unresponsive particles.

SUMMARY.

The problem of determining by means of measurements of electrolytic conductance the permeability of living cells in suspension is considered in some detail and it is pointed out that several factors, usually neglected, have an important influence on the interpretation of such studies. These are:

1. The relative volume and the shape of cells, which are responsive to changes in osmotic pressure and constitution of the surrounding solution. The sources of error in various methods of determining the true volume of red blood cells in a suspension are explained. The hematocrit method appears to be the most reliable method in this case.

2. The proportion of living cells, which is especially to be regarded in the case of suspensions of bacteria. It is shown that this may be very high when appropriate cultural methods are used. The conductance of the dead cells must also be taken into account.

3. The progressive nature of the changes occurring during the course of an experiment. Approximate accuracy may be obtained by proper interpolation.

4. The conductivity of the protoplasm itself, which varies in response to variations is that of the surrounding fluid.

It is emphasized that cells, and in particular red blood cells, are not to be regarded as stable non-conducting particles, but rather as labile and as permeable to electrolytes. It is shown that the available data support this interpretation.

THE EFFECT OF LIGHT ON THE PERMEABILITY OF PARAMECIUM.

By CHARLES PACKARD.

(From the Institute of Cancer Research of Columbia University, New York.)

(Accepted for publication, November 17, 1924.)

Variations in the permeability of plant cells under different conditions of illumination have been described by a number of investigators who employed a variety of methods. Lepeschkin[1] and Tröndle,[2] by the use of the indirect plasmolytic method, showed that cells are relatively permeable when exposed to light of moderate intensity, and much less so when kept in faint light or in darkness. Similar changes have been described by Blackman and Paine[3] who used electrical methods for testing the permeability of cells of *Mimosa pudica*. Hoagland and Davis[4] made chemical analysis of *Nitella* cells after long immersion in salt solutions, and found that the intake of salts was greater when the plants were illuminated than when they were kept in darkness. The general conclusion is that within limits, the permeability of plant cells varies with the amount of illumination. The experiments described below indicate that this is true also for *Paramecium*.

A preliminary test can be made in this way. In the diffuse light of the laboratory, *Paramecia* from a pure line are added to a series of solutions of NH_4OH ($\frac{M}{400}$, $\frac{M}{600}$, $\frac{M}{800}$, etc. made up in tap water). They first give a vigorous avoiding reaction, then swim more and more slowly, until finally they swell and form clear ectoplasmic vesicles, at

[1] Lepeschkin, W. W., *Beitr. bot. Centr.*, 1909, xxiv, 308.

[2] Tröndle, A., *Jahrb. wissensch. Bot.*, 1910, xlviii, 171.

[3] Blackman, V. H., and Paine, S. G., *Ann. Bot.*, 1918, xxxii, 69.

[4] Hoagland, D. R., and Davis, A. R., *J. Gen. Physiol.*, 1923–24, vi, 47.

which time death occurs. In the stronger solutions cytolysis appears in all cells within a few minutes; in the weaker solutions, only after a considerable time. If a similar test is made at night, after the cells have been in darkness for 2 hours or more, it is found that cytolysis and death occur after an appreciably longer time than was found in the first test. The following table shows the result of one such experiment, performed on the same day and at the same temperature.

TABLE I.

Average Time Required to Produce Cytolysis.

NH_4OH solution	$\frac{M}{400}$	$\frac{M}{600}$	$\frac{M}{800}$	$\frac{M}{1000}$
	min.	*min.*	*min.*	*min.*
In light	8	10	14	20
Darkness	8	12	20	30

This method of testing the phenomenon is not suitable for accurate measurement. A better one consists in staining with neutral red the cells which have been exposed to light or darkness, and then transferring them to a weak solution of NH_4OH. As the ammonia enters it changes the red granules to yellow at a rate which is constant under definite conditions. The method has already been briefly described.[5]

The stain is made by adding 2 drops of 0.02 per cent neutral red solution to 10 cc. of tap water, brought to a pH of 7.4 with NaOH. The ammonia solution is made by diluting normal NH_4OH with Sörensen's 7.4 phosphate mixture. The latter should be diluted to approximately $\frac{M}{450}$ since in greater concentrations the cells do not flourish. In place of the phosphate mixture, tap water may be used for dilution, if it is "hard." The results obtained by the use of these two solutions are practically identical, but the latter method is open to objections. The pH of the phosphate-ammonia mixture is 7.8.

In the ammonia all the cells do not lose their red color at the same moment. If the process be watched under a binocular microscope, each cell can be pipetted off as soon as it is decolorized, and the time which has elapsed since it was put into the ammonia, noted. When all

[5] Packard, C., *Biol. Bull.*, 1924, xlvi, 165.

of the cells (25 to 35) have been withdrawn, the average time for decolorizing the entire lot can be calculated. Consecutive tests carried on at the same temperature agree closely, not differing from each other by as much as half a minute.

This method gives consistent results if some variable conditions are kept constant within reasonable limits. One such condition is the temperature. If this is raised, the velocity of color change is likewise increased. A series of tests in which the temperature ranged from 11–31°C. showed that for each increase of 10°C. the velocity of the reaction was approximately doubled. For low temperatures the coefficient is more than 2; it is less than 2 for temperatures above 20°C. Delf[6] and others have shown that the permeability of plant cells varies similarly with changing temperature.

A second variable which should be controlled is the division rate. When cells are rapidly dividing their rate of color change is noticeably higher than when they are dividing slowly. An experiment extending over 2 months in which daily tests were made showed a striking correspondence between the varying division rate and the velocity of decolorizing.

If these two conditions are controlled, the results which follow when *Paramecia* are exposed to lights of different intensities are found to be perfectly consistent. Under moderately bright lights the color change occurs within a few minutes; in darkness, it appears much more slowly.

These phenomena may be explained on the assumption that a high velocity of color change is due to the rapid entrance of ammonia, that is, to a relatively high permeability of the plasma membrane. Conversely, when the color reaction is slow in appearing, it is an indication of a low degree of permeability.

The facts are open to another interpretation. The permeability may remain unaltered but the reaction of the protoplasm may vary. Thus, when the color change appears quickly it may be due to increased alkalinity of the cell substance. However, this view is open to objection. It necessitates the further assumption that when cells are rapidly growing and dividing, or rapidly moving in high

[6] Delf, E. M., *Ann. Bot.*, 1916, xxx, 283.

temperatures, their protoplasm is more alkaline than it is in other circumstances. But since at these times the cells produce large amounts of CO_2 their protoplasm is presumably less alkaline. There is also the possibility that light increases the alkalinity of protoplasm, but evidence on this point is lacking. It appears probable, therefore, that the velocity of color change is a sign of the rate at which ammonia enters the cell and is an indication of the relative permeability of the plasma membrane.

EXPERIMENTAL.

A series of observations was made during the afternoon and evening of the same day in order to determine the effect of gradually decreasing daylight and of darkness. The cells were first tested in diffuse light; in the evening the smallest possible amount of light was allowed to fall on them. Since all of the light which illuminated the cells passed through glass the effective wave lengths were greater than 3,300 Ångström units.

The reaction of the *Paramecia* when first introduced into the NH_4OH solution differs greatly with the intensity of the light which falls on them. In strong light the cells show a vigorous avoiding reaction and swim somewhat more slowly than usual, although they do not die within the time of the experiment. As the daylight fades they show this response less and less, and in the dark they do not show it at all. The time required for the color change to appear grows progressively longer up to a maximum, and remains at this point in cells kept in darkness overnight and tested the next day in a dark room.

The experiment just cited shows clearly that light is an important factor in producing changes in the permeability of *Paramecium* to NH_4OH. Further tests were made to determine what part of the spectrum is chiefly concerned in this action. In these experiments, carried on in a dark room, the cells were first kept in darkness for 2 hours and then exposed to light from a 400 watt lamp at a distance of 25 cm. The following Wratten filters were used as screens; No. 70, red; No. 72, yellow-orange; No. 74, green; No. 76, violet. The cells exposed to white light were protected from the heat of the lamp by a film of $CuCl_2$ solution, 0.5 cm. in thickness. This absorbs some of the

red rays and transmits light of wave lengths up to about 3,600 Ångström units.

Each lot of cells was exposed to light for exactly 2 hours and tested as soon as the exposure ended. An electric fan played continuously on the dishes containing the cells. Frequent thermometer readings showed that the temperature did not vary as much as 1°C. during the tests.

TABLE II.

Effect of Light on Permeability.

$\frac{N}{1000}$ NH_4OH solution. Temperature 22°C.

Time		4.20 p.m. clear.	5.20 p.m. cloudy.	8.20 p.m. dark.	9.20 p.m. dark.	9.20 a.m. dark.
Reaction to NH_4OH		Vigorous avoiding reaction.	Avoiding reaction not so vigorous.	Slight reaction.	No reaction.	No reaction (in dark room overnight).
	min.					
No. of cells decolorized in each min. after entrance into NH_4OH.	1					
	2					
	3	2				
	4	6				
	5	14				
	6	7	4			
	7		6			
	8		11			
	9		14	3	5	6
	10		2	6	9	7
	11			9	12	10
	12			8	9	4
Reaction time		4.9 min.	8.5 min.	10.8 min.	10.7 min.	10.6 min.

There is thus a demonstrable rise in the permeability of *Paramecium* to NH_4OH when the cells are exposed to light from the red end of the spectrum. The intensity of the light transmitted by the red filter is low, being only 0.6 per cent that of the incident light. If it were equal to that of the white light transmitted by the $CuCl_2$ screen this rise would undoubtedly be greater, since permeability rises with increasing intensity up to a certain point.[2] The violet filter transmits but 0.1 per cent of the incident light, yet the rise in permeability in cells exposed to these rays is very marked.

TABLE III.

Effect of Monochromatic Lights.

$\frac{N}{1280}$ NH_4OH solution. Temperature 20°C.

		Dark.	Red.	Yellow-orange.	Green.	Violet.	White.
	min.						
No. of cells decolorized in each min. after entrance into NH_4OH.	1						
	2						
	3						
	4						6
	5					3	7
	6					8	9
	7				3	10	5
	8		3	3	6	15	
	9	6	6	10	6	4	
	10	7	10	16	10		
	11	10	7				
	12	8					
Reaction time.....		10.7 min.	9.8 min.	9.4 min.	8.9 min.	7.2 min.	5.5 min.

TABLE IV.

Effect of Solar Ultra-Violet Light.

$\frac{N}{1280}$ NH_4OH solution. Temperature 19°C.

		Exposed 45 min.		Exposed 90 min.	
		Exposed.	Control.	Exposed.	Control.
	min.				
No. of cells decolorized in each min. after entrance into NH_4OH.	1				
	2			6	
	3	3		9	4
	4	3		5	9
	5	5	3	3	8
	6	10	4		10
	7	3	8		4
	8		6		
	9		4		
Reaction time.....		5.4 min.	7.2 min.	3.2 min.	5.0 min.

In order to test the effect of light waves still shorter than those transmitted by glass (3,300 Ångström units) I made a series of tests in which the dishes containing the *Paramecia*, not covered by glass, were placed out of doors in diffuse north light. In this way the cells

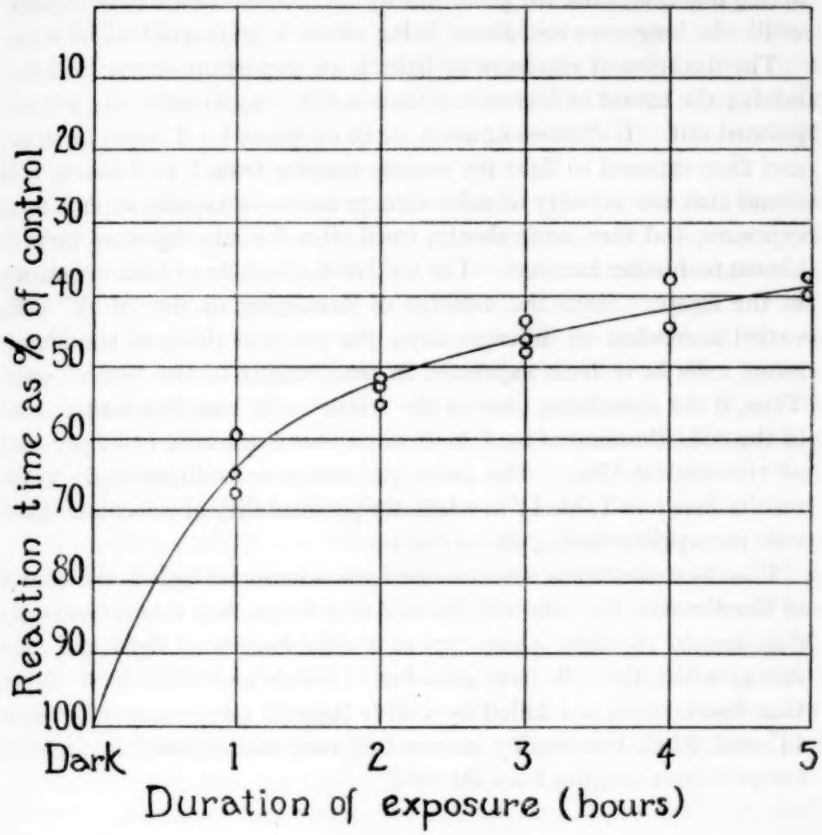


FIG. 1. The effect on permeability of the duration of exposure to light.

received not only the longer waves but also those short waves which are excluded when the experiment is carried on indoors. These shorter waves have lengths from 3,300 to 3,000 Ångström units. Even the shortest of these are readily transmitted by water. Control samples, covered with glass, were tested for comparison. The result of one such series is given in Table IV.

Cells exposed for 30 minutes to 1 hour show a relatively great increase in permeability over the controls. If the exposure is more prolonged this difference tends to become less and less, but it is demonstrable even after 2 hours. Apparently the ultra-violet and visible rays, acting together, raise the permeability up to a maximum very rapidly, while the longer waves, alone, bring about a more gradual increase.

The duration of exposure to light is an important factor in determining the extent of increase of permeability, as Tröndle has already pointed out. If *Paramecia* are kept in darkness for 2 hours or more, and then exposed to light for periods ranging from 1 to 5 hours, it is found that the velocity of color change increases rapidly after 1 hour exposure, and then more slowly, until after 5 hours exposure there is almost no further increase. The results of a number of tests are shown in the figure. Since the velocity of destaining of the "dark" cells varied somewhat on different days, the reaction times of the illuminated cells have been expressed in percentages of the control cells. Thus, if the destaining time of the "dark" cells was 10 minutes, that of the cells illuminated for 1 hour was about 6 minutes, or 60 per cent of the control time. The same phenomenon is illustrated in the results shown in Table IV in which the permeability of cells under glass rose perceptibly during the exposure.

If no heat-absorbing screen is used when artificial light is the source of illumination the cells will die in 3 or 4 hours, but this is obviously due, not to the light alone, but to a combination of light and heat energy which the cells have absorbed. Thiele and Wolf[7] have shown that bacteria are not killed by visible light at temperatures between 14° and 20°C. but readily succumb if they are exposed to light in temperatures ranging from 30–40°C.

DISCUSSION

Light waves produce effects in protoplasm only when they are absorbed by it, and their energy transformed. In the red end of the spectrum, absorption is comparatively slight, nor does it increase materially until the blue and violet are reached. The still shorter ultra-violet rays in sunlight are most readily absorbed, and are most

[7] Thiele, H., and Wolf, K., *Arch. Hyg.*, 1907, lx, 29.

effective in producing a change in permeability. Since wave lengths up to 3,000 Ångström units constitute the normal environment of *Paramecium* and other cells, it is apparent that under ordinary circumstances they can exert no lethal effect, since, as Clark[8] has pointed out, if such rays were lethal all life would have disappeared before this time. However, if light is sufficiently intense, even the longer visible rays may be lethal. Hertel[9] showed that very intense light having a wave length of 4,400 Ångström units causes cytolysis in *Paramecium* in 3 hours. Blood cells are hemolyzed to a slight extent in light filtered through glass.[10]

Since the proportion of energy absorbed by protoplasm increases as the wave length decreases, it is not surprising to find that ultra-violet rays are lethal. According to Hertel[9] when *Paramecium* is exposed to rays shorter than those found in sunlight it quickly cytolyzes. Tchahotine[11] observed that when a narrow pencil of ultra-violet rays is directed on a sea urchin egg, the point of illumination shows a greatly increased permeability to water. Henri[12] found that a lethal action on bacteria is just noticeable at 3,097 Ångström units, while Browning and Russ[13] determined that the most pronounced bactericidal effect occurs between 2,940 and 2,380 Ångström units.

Since both sunlight and the rays of the ultra-violet region penetrate the entire cell it is probable that all parts of the cell are affected; that is, the changes in the plasma membrane are only one expression of the general effect produced. But the extremely short rays are entirely absorbed in the membrane not reaching the interior of the cell. This was pointed out by Hughes and Bovie[14] who exposed *Paramecium* to wave lengths of 1,600 Ångström units. In many cells cytolysis and death resulted, but the cells which escaped destruction continued to divide, indicating, according to the authors, that the rays did not reach the nucleus.

[8] Clark, J. H., *Physiol. Rev.*, 1922, ii, 277.

[9] Hertel, E., *Z. allg. Physiol.*, 1905, v, 95.

[10] Hasselbalch, K. A., *Biochem. Z.*, 1909, xix, 435.

[11] Tchahotine, S., *Compt. rend. Soc. biol.*, 1921, lxxxiv, 464.

[12] Henri, V., *Compt. rend. Acad.*, 1912, clv, 315.

[13] Browning, C. H., and Russ, S., *Proc. Roy. Soc. London, Series B*, 1917–19, xc, 33.

[14] Hughes, D. M., and Bovie, W. T., *J. Med. Research*, 1918–19, xxxix, 233.

Changes in permeability are also brought about by the infra-red or heat radiation, as has already been pointed out. There is evidence that at the other end of the spectrum, the shortest waves, or x-rays, are absorbed and that the transformation of this absorbed energy is accompanied by a rise in permeability.

It thus appears that when sufficient energy is absorbed from any part of the spectrum the physiological condition of the cell is altered, and that one expression of such change is to be found in a change in the plasma membrane. Some investigations have been made to determine the chemical changes which occur during exposure to light. Clark[8] states that there is some evidence that lipoids are rendered more soluble, while Young[15] finds that proteins are coagulated by sunlight. However, very little is known about this important topic.

Finally, these experiments demonstrate the fact that *Paramecium* is extremely sensitive to some external factors which are not usually taken into account. If these cells are to be used in physiological experiments, the amount of illumination which they receive must be controlled.

SUMMARY.

1. The permeability of *Paramecium* to NH_4OH is greater when the cells are exposed to light than it is when they are in darkness.

2. This change can be demonstrated in cells exposed to monochromatic red light, though it is small. It becomes greater as the wave lengths shorten, and is greatest in the near ultra-violet.

3. The permeability increases as the duration of exposure to light is prolonged.

4. These experiments demonstrate the necessity of controlling the illumination when using *Paramecium* in physiological tests.

[15] Young, E. G., *Proc. Roy. Soc. London, Series B*, 1922, xciii, 235.

THE KINETICS OF THE DECOMPOSITION OF PEROXIDE BY CATALASE.

By JOHN H. NORTHROP.

(From the Laboratories of The Rockefeller Institute for Medical Research.)

(Accepted for publication, November 21, 1924.)

The study of the kinetics of catalase, as of most other enzymes, has led to contradictory results. It was found by Senter[1] that the reaction was monomolecular with dilute peroxide and low temperature, but that under other conditions the reaction could not be fitted to the monomolecular formula. Senter ascribed this anomaly to the destruction of the enzyme and limited his experiments to conditions under which the monomolecular formula held. Sörensen,[2] Michaelis and Pechstein,[3] Evans,[4] and others followed the reaction at higher temperature and with more concentrated peroxide, and confirmed Senter's results that under these conditions the reaction was not monomolecular. They were unable to derive any satisfactory mechanism for the reaction. Yamasaki[5] concluded that the enzyme was decomposing at a rate that was proportional to the rate of decomposition of the peroxide and could account for his results fairly well on this basis. He was unable, however, to give any mechanism for this relation. A thorough experimental study of the reaction has recently been made by Morgulis,[6] who also found that at 20°C. and high peroxide concentration the reaction was in general not monomolecular. Part of his results could be calculated by the bimolecular formula but a large part of them could not be accounted for. Morgulis' experiments showed that the enzyme

[1] Senter, G., *Z. physik. Chem.*, 1903, xliv, 257.
[2] Sörensen, S. P. L., *Biochem. Z.*, 1909, xxi, 131.
[3] Michaelis, L., and Pechstein, H., *Biochem. Z.*, 1913, liii, 320.
[4] Evans, C. A. L., *Biochem. J.*, 1907, ii, 133.
[5] Yamasaki, E., *Sc. Rep. Tohoku Imp. Univ.*, 1920, ix, 13.
[6] Morgulis, S., *J. Biol. Chem.*, 1921, xlvii, 341.

was being rapidly inactivated under the conditions of his experiments. In some experiments on the kinetics of trypsin the writer has found similar conditions and has been able to show experimentally that the inactivation of the enzyme is the cause of the anomalous results.[7] It has been found that the formulation derived for the trypsin experiments also agrees very well with the results of Morgulis and the present paper is a recalculation of Morgulis' results on this basis.

Experimental Conditions and General Results.

Morgulis followed the course of the reaction by noting the volume of oxygen given off. The reaction mixture was shaken to avoid supersaturation. The temperature was 20°C. The catalase was prepared from liver. Owing to the method of measurement, the total amount of oxygen was recorded and not the amount per unit volume of the solution, as is usually done. For use in this paper the results have been calculated to unit volume.

Effect of Varying the Concentration of Catalase.

The results of increasing the concentration of catalase with the same concentration of peroxide are given in Table I. It is evident that the time required to produce a constant amount of oxygen decreases much more rapidly than the concentration of enzyme increases. The value of Qt, therefore, decreases rapidly as the enzyme increases, which is contradictory to the result predicted for a catalyzed monomolecular reaction.

In Table II the amounts of oxygen liberated after equal time intervals with different concentration of catalase are given. The table shows that there is a direct proportionality between the amount of oxygen and the concentration of catalase. This result is the opposite of what is usually obtained with enzymes.

Effect of Varying the Peroxide Concentration.

A summary of the experiments in which the concentration of peroxide was varied, keeping the catalase constant, is given in Table

[7] Northrop, J. H., *J. Gen. Physiol.*, 1923–24, vi, 429.

TABLE I.

H_2O_2 = 0.32 M. (Morgulis, p. 359.)

Relative concentration of catalase.	Time to form 25 cc. O_2.	Qt
cc.	*min.*	
3	4.25	12.7
4	1.90	7.6
5	1.50	7.5
6	0.75	4.5
7	0.45	3.1

TABLE II.

Oxygen Evolved at Equal Time Intervals.

H_2O_2 = 0.20 M. (Morgulis, p. 359.)

Relative concentration of catalase.	O_2 after		$\frac{O_2}{\text{catalase}}$ (after 20 min.)
	10 min.	20 min.	
cc.	*cc.*	*cc.*	
6.75	80	102	15
5.63	69	91	16
4.5	55	76	17
3.75	47	63.5	17
3.0	33.6	46.4	16

TABLE III.

Concentration of H_2O_2.

Catalase constant (5 cc.). (Morgulis, p. 359.)

Concentration of H_2O_2.	Time to form 25 cc. O_2.	O_2 liberated in 10 min.
M	*min.*	*cc.*
0.16	1.2	81.6
0.20	1.55	85.6
0.24	1.70	82.5
0.28	1.85	81.6
0.32	1.80	81.6
0.36	1.85	82.9
0.40	2.35	69.4

III. The results show that the time required to liberate a constant amount of oxygen increases slightly as the peroxide increases, whereas the amount of oxygen liberated in a constant time interval is independent of the peroxide concentration over a considerable range. This result confirms the statement made above that the rate of reaction is independent of the peroxide concentration in this range and that the enzyme is decreasing as a function of time and not as a function of the amount of oxygen produced. This result was obtained experimentally by Michaelis and Pechstein, who found that the reaction as followed by the decrease in peroxide was identical whether or not the oxygen was removed as formed or allowed to accumulate in the solution. The fact that the reaction was independent of the peroxide concentration in concentrations over about 0.1 M was noted by Senter. This is a common phenomenon in enzyme reactions and may be accounted for either by assuming that the enzyme forms a compound with the substrate or that there is an equilibrium between the substrate and the solvent, so that the "active" concentration of substrate does not increase as does the apparent concentration. Yamasaki has also been able to account for this effect in the case of urease by considering the reaction as consisting of three consecutive steps. The same phenomenon is known in trypsin hydrolysis and the writer has been able to show experimentally that it is not due to the formation of a compound between the enzyme and substrate.[8]

Relation between the Concentration of Catalase and the Total Amount of Oxygen Liberated.

The results of the experiments in which the concentration of catalase was varied are given in Table IV. The table shows that the total amount of oxygen liberated is directly proportional to the concentration of catalase (provided, of course, that there is an excess of peroxide). This result is the exact opposite of that expected for a catalytic reaction, since the end-result should be independent of the concentration of catalyst.

[8] Northrop, J. H., *J. Gen. Physiol.*, 1923–24, vi, 239, 337.

Concentration of Peroxide and Total Amount of Oxygen Liberated.

The relation between the concentration of peroxide and the total amount of oxygen liberated is shown in Fig. 1. It shows that the total amount of oxygen liberated increases with the concentration of peroxide up to about 0.32 M and then decreases and that there is no constant percentage decomposition. This result also applies only to the case where there is excess peroxide.

TABLE IV.

(Morgulis, p. 368.)

$H_2O_2 \Rightarrow$ 112 cc. O_2.			$H_2O_2 \Rightarrow$ 181 cc. O_2.		
Catalase.	Total O_2.	$\frac{O_2}{\text{cc. catalase}}$	Catalase.	Total O_2.	$\frac{O_2}{\text{cc. catalase}}$
cc.	*cc.*		*cc.*	*cc.*	
6.75	112	17	7	180.7	25.8
5.63	104	18	6	158.0	26.3
4.5	88	17	5	122.7	24.5
3.75	70	19	4	106.0	26.5
3.0	57	19	3	71.0	23.9

Summary of General Results.

The results of the foregoing experiments may be summarized as follows:

Peroxide Constant and in Excess, and Catalase Varied.

1. The amount of oxygen liberated after a given time is directly proportional to the concentration of catalase

2. The total amount of oxygen liberated is directly proportional to the concentration of catalase.

3. There is no simple relation between the time required to liberate a constant amount of oxygen and the concentration of catalase. The time decreases much more rapidly than the catalase concentration increases.

Catalase Constant and Peroxide Varied. Peroxide Always in Excess.

1. The amount of oxygen liberated after a constant time interval is independent of the peroxide concentration within a wide range.

2. The total amount of oxygen liberated increases up to a concentration of peroxide of 0.32 M and then decreases.

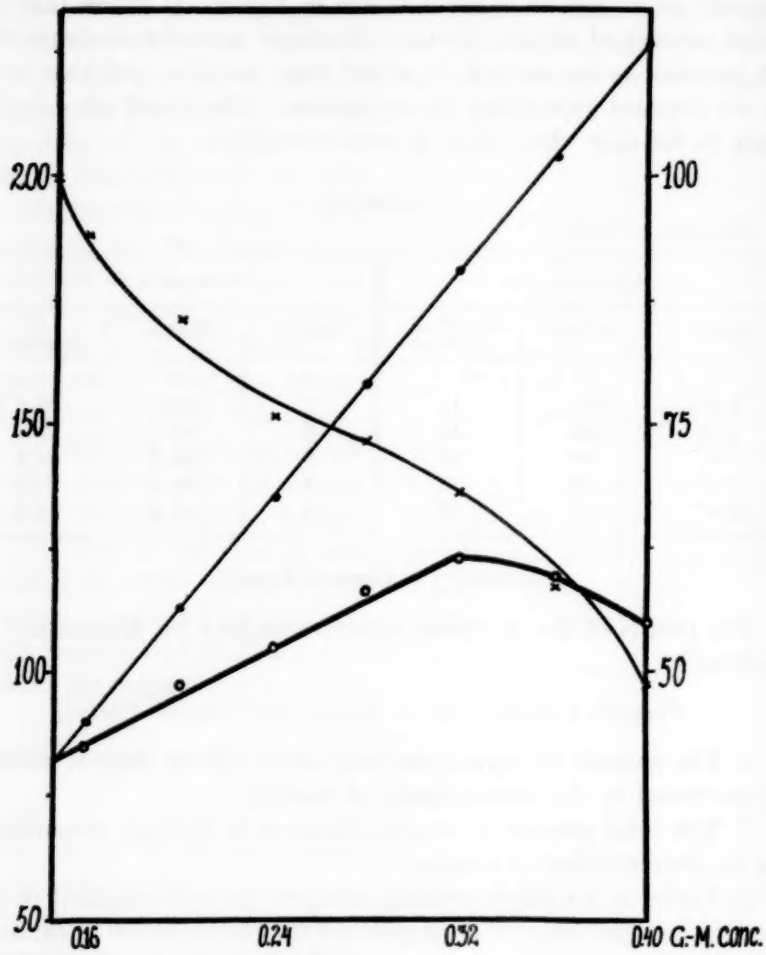


FIG. 1. Effect of increasing quantities of hydrogen peroxide on the catalase reaction. ●—● cc. of oxygen available. o—o cc. of oxygen liberated. ×—× per cent of hydrogen peroxide decomposed. (After Morgulis.)

3. The time required to liberate a constant amount of oxygen increases as the peroxide increases.

These results show definitely that under these conditions the reaction is not monomolecular with respect to the peroxide and catalyzed by the enzyme, since in that case the end-result would be independent of the concentration of enzyme and not directly proportional to it as is the experimental result. They also show that the reaction is not bimolecular nor does the catalase enter into it in any stoichiometric way, since the total amount of oxygen formed with a constant amount of catalase varies with the concentration of peroxide.

Formulation of the Reaction.

According to classical physical chemistry the general formula for the rate of reaction between any two molecules is

$$-\frac{dS}{dt} = KES$$

in which E is the concentration of (in this case) catalase at the time t, and S is the concentration of peroxide at the time t. If the catalase behaves as a typical catalyst its concentration will not change during the course of the reaction and E becomes a constant. The equation then becomes $-\frac{dS}{dt} = K'S$ or, if x is the amount of substrate decomposed, $\frac{dx}{dt} = K'(S_0 - x)$ which is the ordinary monomolecular formula. This formula was found to hold for the present reaction at low temperatures and low concentration of peroxide by Senter. The results of Morgulis, however, show that (1) the concentration of enzyme is rapidly decreasing as a function of the time, and (2) the reaction rate is independent of the initial peroxide concentration. The formula therefore becomes

$$\frac{dx}{dt} = kSE$$

in which k is the velocity constant of the reaction, S is a constant representing the "active" concentration of peroxide (and independent of the concentration used provided the latter is above 0.1 M), and E is the concentration of catalase at time t. The simplest assumption

in regard to the catalase would be that it was decomposing monomolecularly itself. The concentration E at any time would then be $E_t = E_0 e^{-Kt}$ in which e is the base of the natural logarithms and K is the constant for the decomposition of the enzyme. The formulation of the entire reaction then becomes

$$\frac{dx}{dt} = kSE_0 e^{-Kt}$$

On integrating this equation it becomes

$$x = \frac{SkE_0}{K}(1 - e^{-Kt}) \tag{1}$$

It may be seen that this equation predicts qualitatively the experimental results obtained by Morgulis, and summarized on page 377. For instance, if $t = \infty$, $x = \frac{SkE_0}{K}$, that is the total amount of oxygen formed is proportional to the amount of catalase taken.

If S and t are the same in two experiments, $\frac{x}{x'} = \frac{E}{E'}$, *i.e.* the amount of oxygen produced in a given time is proportional to the amount of catalase taken.

If E_0 and t are the same, but the peroxide concentration is varied, the same amount of oxygen will be liberated since there is no term in the equation for the original concentration of peroxide.

If E is varied and the time noted to liberate x cc. of oxygen, *i.e.* $x = x'$, the time will decrease more rapidly than the enzyme increases, since the formula then becomes

$$\frac{E'}{E} = \frac{1 - e^{-Kt}}{1 - e^{-Kt'}}$$

This type of result has been taken as evidence that the rate of reaction is some exponential function of the enzyme concentration and that therefore the enzyme is adsorbed by the substrate or *vice versa*. The above derivation shows that it is simply the result of the fact that the enzyme is decreasing as a function of time and not as a function of the products of the reaction.

As will be seen below the value of K varies with the original concentration of peroxide. The mechanism of this effect and of the fact that the reaction rate is independent of this value is not taken into account in the above formula.

In order to apply the formula to the time curves of the reaction it may be further simplified as follows:

Since SkE_0 and K are constant for any one experiment, the term $\frac{SkE_0}{K}$ will be constant and may be made equal to A; substituting in (1) $x = A - Ae^{-Kt}$ or

$$K = \frac{1}{t} 1n \frac{A}{A - x}$$

which is again the form of a monomolecular reaction. It must be noted, however, that A does not refer to the peroxide but to the enzyme. It is proportional to the total amount of oxygen liberated, therefore, and not, as would ordinarily be the case, to the total amount of substrate present. K is the constant of decomposition of the enzyme and not the reaction constant. It may vary, therefore, with the peroxide concentration but will be independent of the enzyme concentration which is just the opposite of the behavior of the ordinary monomolecular constant.

Morgulis' experiments have been recalculated with this formula and found to agree very well. The value of A has been taken directly in the cases where a maximum value was reached. In other cases it was obtained by graphic extrapolation; which is of course a rather uncertain method. The results with varying pH, catalase concentration, and peroxide concentration are given in Tables V to VII. It will be seen in every case that the value of K is constant within the experimental error.

Effect of Various Factors on the Value of K.

The values obtained for K, which is the measure of the rate of inactivation of the enzyme, at different degrees of acidity with the same concentration of enzyme and peroxide, are given in Tables V and VIII. Apparently the effect of the pH is not on the main reaction but on the inactivation of the enzyme.

TABLE V.

Rate at Different pH.

H_2O_2 = 0.31 M. Total O_2 = 173 cc. 5 cc. catalase. (Morgulis, pp. 345–346.)

pH	t	$A-x$	K	pH	t	$A-x$	K
	min.	*cc.*			*min.*	*cc.*	
5.2	0	98		7.2	0	133	
	5	47	0.064		5	72.7	0.053
	10	23	0.063		10	43.1	0.049
	15	9	0.068		15	24.8	0.048
	20	4	0.069		20	13.6	0.049
	23	2.7	0.068		25	6.8	0.051
					31	3.0	0.053
5.8	0	122		7.5	0	138	
	5	59.5	0.062		5	77.9	0.051
	10	31.8	0.058		10	48.4	0.045
	15	16.8	0.058		15	30.0	0.045
	20	8.7	0.058		20	17.4	0.048
	25	4.9	0.054		25	10.0	0.045
	31	2.3	0.057		30	5.2	0.047
					35	2.8	0.049
6.4	0	134		8.3	0	138	
	5	71.0	0.055		5	78.9	0.048
	10	40.4	0.0523		10	51.4	0.043
	15	20.6	0.054		15	30.2	0.044
	20	9.8	0.057		20	18.0	0.044
	25	5.7	0.054		25	10.4	0.049
	29	2.8	0.057		30	5.0	0.048
	33	1.3	0.060		35	1.9	0.053
7.0	0	140					
	5	74.9	0.054				
	10	44.7	0.049				
	15	25.7	0.049				
	20	14.8	0.049				
	25	9.1	0.052				
	29	6.1	0.047				
	34	3.9	0.046				

Tables VII and IX show the relation of the peroxide concentration to the value of K. Increasing the peroxide up to 0.4 M decreases the rate of inactivation of the enzyme.[9] It is evident, there-

TABLE VI.

Varying Concentrations of Catalase.

H_2O_2 = 112 cc. O_2. (Morgulis, p. 368.)

Catalase.	t	$A-x$	K	Catalase.	t	$A-x$	K
cc.	min.	cc.		cc.	min.	cc.	
6.75	0	113		3.75	0	71	
	5	56.4	0.060				
	10	33.0	0.053		10	23.9	0.047
	15	19.0	0.052				
	20	10.9	0.051		20	7.5	0.048
	25	6.7	0.050				
	30	4.2	0.047		30	1.8	0.053
	40	1.2	0.050		40	1.0	0.046
5.63	0	105		3.0	0	59	
	5	56.0	0.054				
	10	35.7	0.047		10	24.2	0.0387
	15	22.9	0.045				
	20	12.5	0.046		20	12.6	0.034
	25	7.5	0.045				
	30	4.0	0.047		30	5.8	0.033
	40	1.3	0.053		40	2.6	0.034
					45	1.8	0.034
4.5	0	89					
	5	51.6	0.046				
	10	33.9	0.042				
	15	21.6	0.041				
	20	12.9	0.042				
	25	8.3	0.041				
	30	5.3	0.041				
	35	3.2	0.040				
	45	1.0	0.043				

fore, that, as Morgulis stated, the enzyme is not oxidized by the peroxide. Further evidence that this is the case is shown by the

[9] In some unpublished experiments kindly furnished the writer by Professor Morgulis the rate of inactivation was found to be independent of the peroxide concentration. Professor Morgulis also kindly furnished the cut for Fig. 1.

fact that the value of K for any one reaction is constant. The concentration of peroxide is, however, decreasing. The inactivation of

TABLE VII.

Varying Concentrations of Peroxide.

Catalase 5 cc. Total volume 50 cc. (Morgulis, p. 364.)

Concentration of H_2O_2.	t	$A-x$	K	Concentration of H_2O_2.	t	$A-x$	K
M	*min.*	*cc.*		M	*min.*	*cc.*	
0.16	0	90		0.32	0	127	
	5	25.6	0.109		5	70.5	0.051
	10	8.4	0.104		10	45.4	0.045
	12	5.0	0.103		15	27.5	0.044
					20	16.4	0.039
					25	9.0	0.060
					30	5.0	0.043
0.20	0	100		0.36	0	125	
	5	36.9	0.087		5	69	0.051
	10	14.4	0.084		10	42	0.048
	15	4.9	0.087		15	24.5	0.053
	18	3.0	0.085		20	14	0.0475
					25	8	0.047
					30	6	0.043
0.24	0	106		0.40	0	115	
	5	47.5	0.070		5	67.3	0.046
	10	23.5	0.065		10	45.6	0.040
	15	9.5	0.069		15	28.4	0.040
	20	2.7	0.078		20	17.2	0.041
	24	1.5	0.077		25	9.8	0.043
					30	5.9	0.043
					33	5.3	0.041
0.28	0	120					
	5	63.3	0.056				
	10	38.4	0.050				
	15	20.5	0.051				
	20	9.6	0.055				
	25	4.8	0.056				
	27	4.1	0.054				

the enzyme, therefore, cannot be due to the peroxide. It seems probable that the stabilizing effect noted is due to an impurity pres-

ent in the peroxide. In higher concentration, however, the inactivation is increased rapidly and is here probably due to direct oxidation. Under these conditions the constants drop.

TABLE VIII.

Effect of pH on Value of K.

pH	K
5.2	0.068
5.8	0.057
6.4	0.055
7.0	0.049
7.2	0.049
7.5	0.049
8.3	0.047

TABLE IX.

Rate of Decomposition and Concentration of H_2O_2.

(Morgulis, p. 364.)

Original concentration of H_2O_2.	K	K × concentration H_2O_2
0.16	0.105	0.0168
0.20	0.086	0.0172
0.24	0.072	0.0172
0.28	0.053	0.0148
0.32	0.048	0.0153
0.36	0.048	0.0172
0.40	0.042	0.0168
2.4	0.7–0.5	

TABLE X.

(*Cf.* Table VI.)

Relative concentration of catalase.	K
6.75	0.051
5.63	0.048
4.5	0.042
3.75	0.048
3.0	0.035

Table X shows that the rate of inactivation is nearly independent of the enzyme concentration as it should be if the decomposition of the enzyme is really monomolecular.

The mechanism upon which the equation used is based furnishes, therefore, a simple explanation for the experimental results obtained by Morgulis. The reaction proceeds in accordance with the laws of mass action under these conditions just as under the conditions studied by Senter. In the former case, however, the reaction rate is determined almost entirely by the destruction of the enzyme, whereas in Senter's experiments it was determined by the decomposition of the peroxide. It is evident that between the two extreme conditions there will be a zone where neither formula will hold accurately but where both substrate and enzyme would have to be considered as variables. A case similar to this was considered by Tammann working with emulsin, and has been found by the writer to be true for trypsin.[10] If the catalase is just sufficient to decompose nearly all the peroxide the reaction will be monomolecular with respect to each, *i.e.* $\frac{dx}{dt} = C(A - x)(B - x)$, in which A is the concentration of catalase, and B is the concentration of peroxide. Since under these conditions $A = B$, the reaction follows the bimolecular course as Morgulis pointed out.

The experiments of Yamasaki were made with a vegetable catalase and differ somewhat from those considered here. They cannot be calculated by the present formula since the enzyme does not decompose monomolecularly, but becomes progressively more stable. Yamasaki has taken this into account by setting the rate of inactivation equal to the substrate concentration. This method leads to an equation which fits the experiments quite well, but which contains several constants. It seems possible that the difficulty is due to the fact that Yamasaki did not regulate the pH of the solution.

Michaelis and Pechstein's, and Sörensen's results are in general agreement with those discussed here except that they did not use such high concentrations of peroxide. The variation in the peroxide concentration must therefore be considered.

SUMMARY.

It has been shown that the experimental results obtained by Morgulis in a study of the decomposition of hydrogen peroxide by

[10] Northrop, J. H., *J. Gen. Physiol.*, 1923–24, vi, 439.

liver catalase at 20°C. and in the presence of an excess of a relatively high concentration of peroxide are quantitatively accounted for by the following mechanisms.

1. The rate of formation of oxygen is independent of the peroxide concentration provided this is greater than about 0.10 M.

2. The rate of decomposition of the peroxide is proportional at any time to the concentration of catalase present.

3. The catalase undergoes spontaneous monomolecular decomposition during the reaction. This inactivation is independent of the concentration of catalase and inversely proportional to the original concentration of peroxide up to 0.4 M. In very high concentrations of peroxide the inactivation rate increases.

4. The following equation can be derived from the above assumptions and has been found to fit the experiments accurately.

$$K = \frac{1}{t} \log \frac{A}{A - x}$$

in which x is the amount of oxygen liberated at the time t, A *is the total amount of oxygen liberated* (not the total amount available), and K is the inactivation constant of the enzyme.

ELECTROENDOSMOSIS THROUGH MAMMALIAN SEROUS MEMBRANES.*

I. The Hydrogen Ion Reversal Point with Buffers Containing Polyvalent Anions.

By STUART MUDD.

(*From the Laboratories of The Rockefeller Institute for Medical Research.*)

Plates 1 and 2.

(Accepted for publication, November 25, 1924.)

It is shown in this communication that if an electric current is passed through a mammalian serous membrane bathed in dilute buffer solution, the solution is caused to stream by electroendosmosis through the membrane toward the cathode at reactions alkaline or near neutrality, but toward the anode with buffers of moderately acid reaction. This paper and others to follow are intended to establish certain of the conditions of such electroendosmotic fluid transport across mammalian membranes when a current is introduced from an external source. Certain interesting physicochemical characteristics of mammalian serosæ have emerged in the course of the present experiments. A physiologically balanced buffer isotonic with the tissue fluid is described. The problem of bioelectric currents as a possible source of electrokinetic effects is reserved for future study.

Method and Materials.

Apparatus.—The simple apparatus adopted after a number of trials is shown in Fig. 1. The tissue membrane, either in place in the animal or excised, is applied to the mouth of a glass electrode vessel provided with a side tube graduated in millimeters. The aperture it covers is 9 mm. in internal diameter. The upper end of the electrode vessel is fitted with a short piece of rubber tubing through which the vessel may be filled and washed; the tubing is clamped off during a run

* A preliminary report of this study has recently appeared (Mudd, S., *Science*, 1924, lx, 527).

with a small hemostatic forceps. The side tube is tilted slightly upward from the horizontal; and into it is sealed a platinum electrode connected on the outside of the tube with a length of copper wire. This copper connecting wire is coiled and sealed to the outside of the tube with De Khotinsky cement.[1] The other electrode is a glass tube containing a wire immersed in buffer solution over a plug of buffered

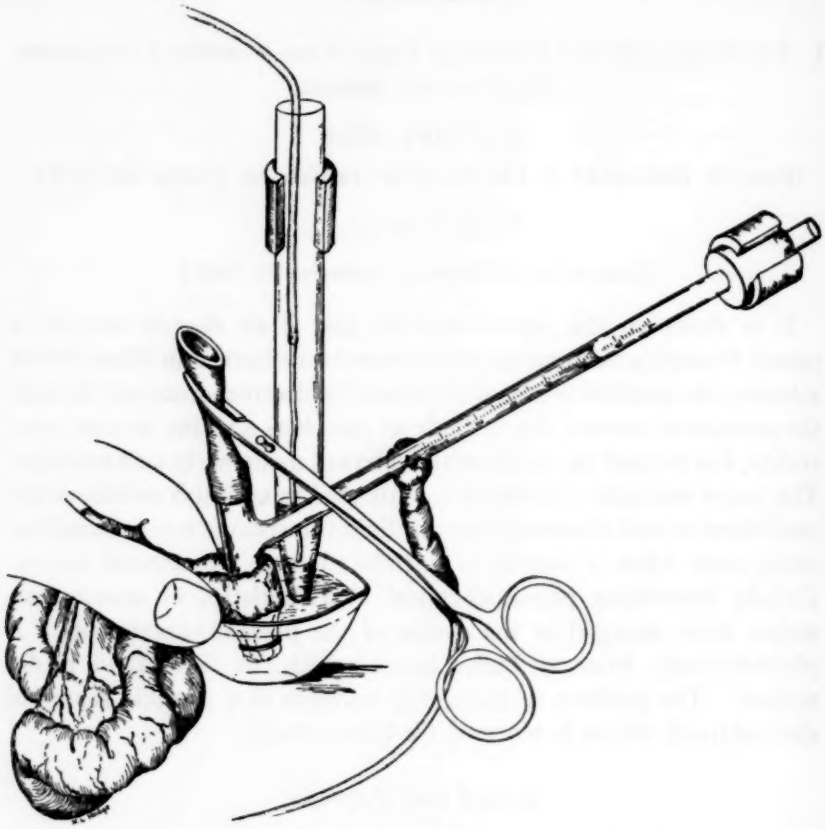


FIG. 1. Arrangement for experiment on electroendosmosis through mesentery of a living animal. For description see text.

agar. During the observations the membrane and agar electrode are kept in a crystallizing dish full of the same buffer as that inside the electrode vessel. The 220 volt line is used as source of electromotive force.

[1] This device, due to Dr. G. Failla, serves to protect the vessel from the otherwise frequent accident of having the platinum wire break off close to the glass seal.

The membrane is held in place over the mouth of the electrode vessel by means of a circular rubber band cut from a piece of rubber tubing. The rubber band is applied with the aid of a device made from a Halstead curved mosquito forceps (Fig. 2). The inside of the jaws of the forceps are filed smooth and a third jaw is fastened to the forceps by means of a machine screw and solder. A shoulderis filed into each jaw just below the tip, and the rubber is stretched over the shoulders thus formed whence it can readily be slipped off into the groove above the mouth of the electrode vessel.

Structure of Serous Membranes.—The mammalian serosæ of the major body cavities vary considerably in detail of structure even at different points in the same cavity. Practically the whole of the parietal pericardium was found to be

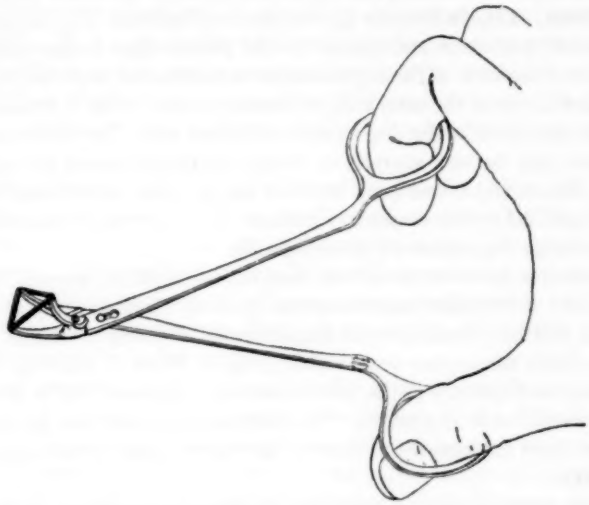


FIG. 2. Device for applying rubber band. See text.

serviceable for the experiments. A fibrous sheet of pleura extending between the caudal tip of the pericardium and the diaphragm was also well adapted for experiments and was regularly employed. Experiments with the peritoneal serosa were usually performed on the mesentery of the small intestine, much less frequently on the mesocolon or the mesocecum.

All of the membranes mentioned are covered on their two surfaces with a single layer of pavement mesothelial cells. The free surfaces of these mesothelial cells are described[2] as in close but not in such perfect apposition but that fine intercellular crevices may be demonstrated and particles may be forced between the

[2] MacCallum, W. G., *Bull. Johns Hopkins Hosp.*, 1903, xiv, 105.

cells. The larger preformed openings or stomata of earlier authors have apparently been shown to be non-existent by later work.[2,3]

Next to the mesothelium of one or both sides of the membrane lies a fairly dense layer of fibrous connective tissue as shown in Fig. 1, Plate 1, and Fig. 4, Plate 2, in which cells are scarce. The collagen fiber bundles lie parallel to the mesothelial surface and elastin fibers are woven between the collagen bundles. The thicker membranes, especially the pericardia, often have a definite layer of areolar connective tissue bounded on one side by mesothelium, or a thin layer of connective tissue, and on the other by the dense fibrous layer just mentioned. Fat is deposited in this areolar layer of the pericardium (Fig. 1, Plate 1), and also in the mesentery (Fig. 3, Plate 2), especially about the larger blood vessels. If the animal is obese the windows of membrane between the blood vessels also contain fat deposit. Elastin fibers in the sections studied (cat, dog, and rabbit) were relatively more numerous and coarser in the pleuræ than in the pericardia. In the lean pleura sections of the region under consideration, such fibers made up a considerable fraction of the total bulk of fibrous material, (Fig. 2, Plate 1), amounting at some situations in the dog to close to 50 per cent. In the mesentery, also, elastic fibers may be seen sparsely or thickly scattered among the collagen bundles of the fibrous tissue and lying between the fat cells, where these are present. In sections parallel to the mesentery surface, a loose meshed elastic network has been seen through the pavement epithelial cells.

The variability in structure of the membranes needs emphasis. Even inthe several regions of one microscopic section, the total thickness, the amount of fat deposit, the relative proportions of dense fibrous and areolar tissue, and of collagen and elastin fibers may vary widely (Fig. 2, Plate 1, and Fig. 3, Plate 2). The thickness and strength of the membranes are, in general, less in the cat than in the dog, and still less in the rabbit. The pericardium of any one species is thicker and stronger than the pleura—at least in the regions used—much more so than is the mesentery.

During the accumulation or resorption of serous transudates, or in an electroendosmotic experiment liquid must pass through the capillary interstices of the membranes, between (and through?) the mesothelial cells and the connective tissue fibers, and between the fat cells. Under natural conditions the liquid has to pass only part way through the membrane whereas under the experimental ones it passes through both leaves.

Buffer Solutions.—In the earlier experiments reported below, the citric acid-sodium phosphate buffer described by McIlvaine[4] was used. In electroendosmotic experiments, however, it is not convenient to work with a buffer of such low resistance. Stock mixtures were therefore made up as tabulated by Clark[5] and

[3] Cunningham, R. S., *Am. J. Physiol.*, 1922, lxii, 248.

[4] McIlvaine, T. C., *J. Biol. Chem.*, 1921, xlix, 183.

[5] Clark, W. M., The determination of hydrogen ions, Baltimore, 2nd edition, 1922, 116.

diluted, before use, with distilled water so as to contain 1 volume of stock buffer in 25 volumes of solution. These dilute buffer mixtures have been designated below as "McIlvaine's." The pH was shifted to the alkaline side by the dilution, from 1.8 to 6.6 pH units. The pH values of the dilute mixtures were determined as routine electrometrically and in duplicate. The hydrogen electrodes were standardized at intervals against N/10 HCl the pH of which at 25°C. was taken as 1.037.

The molarity of a dilute buffer at pH = 5.06 was 0.00587 M. In the acid range the molar concentration decreased to 0.00408 M at pH = 2.9 approximately; in the alkaline range it increased to 0.00763 M at pH = 7.7 approximately. The dilute buffers were accordingly of only about $\frac{1}{25}$ the molarity of the blood in the reversal point region, and hence very hypotonic for the tissues studied. Furthermore, these buffers contained only $H^{\cdot}$ and $Na^{\cdot}$ as cations and were thus unbalanced physiologically. That such a buffer solution is toxic follows from general biological experience and may readily be demonstrated by applying it to a living mesentery. The tissue at once loses its transparency and becomes clouded. The problem of preparing a suitable isotonic, physiologically balanced buffer was accordingly undertaken.

Isotonic, Physiologically Balanced Buffers.—The sodium citrate and phosphate mixture was retained and potassium chloride and calcium chloride were added in such proportions as to make the final ratio of Na : K: Ca the same as in Ringer's solution. The $NaHCO_3$ of Ringer's solution was omitted as unsuitable in a buffer for use where control of CO_2 tension was not practicable. The total electrolyte concentration adopted was about $\frac{1}{25}$ that of the blood, thus making the specific resistance of the buffer high.

The osmotic pressure of the balanced buffer mixture was, in the preliminary work, adjusted to approximately that of the tissue fluids by the addition of glucose. The use of glucose, however, necessitated elaborate precautions for sterility. Oxidation of the glucose in alkaline solution[6] limited the range of usefulness of the buffer and even in the acid range the potential of the H electrodes in measuring the pH was never quite so stable or reproducible as in the absence of the sugar. To avoid these difficulties, glycerol was at length substituted for glucose, at the suggestion of Dr. P. A. Levene. The total molar concentration of the buffer was brought to approximate equivalence wth 5.4 per cent dextrose solution, which Rous and Turner found isotonic with mammalian red blood cells.[7] This glycerolated, isotonic, physiologically balanced buffer (I. P. B. B. No. 3) proved very satisfactory. This solution or Ringer's solution, when left for 1 or 2 minutes in contact with living dog mesentery, caused a slight opacity; but distilled water or dilute McIlvaine's buffer caused a much greater opacity.

---

[6] It could be shown that the hydrogen electrode did not even faithfully represent the pH values of an *alkaline* glucose solution.

[7] Rous, P., and Turner, J. R., *J. Exp. Med.*, 1915, xxiii, 227.

A titration curve against N/100 HCl made isotonic with glycerol was determined electrometrically from pH = 8.1 (pure buffer) to pH = 2.85 (buffer 5 volumes, N/100 isotonic HCl 6 volumes). Fresh buffer mixtures were thereafter made according to the titration curve, and checked at the critical reactions with the hydrogen electrode.

The formula of the glycerolated, isotonic, physiologically balanced buffer was as follows:

Isotonic, Physiologically Balanced Buffer No. 3.

	gm.
Na_2HPO_4 (Merck's anhydrous)	1.895
$Na_3C_6H_5O_7 \cdot 5\frac{1}{2}H_2O$ (Powers-Weightman-Rosengarten Co. analytical)	4.765
KCl (Merck's Blue label reagent)	0.034
$CaCl_2$ (Powers-Weightman-Rosengarten Co. anhydrous)	0.029
Glycerol (Powers-Weightman-Rosengarten Co. analytical—95 per cent glycerol)	152.0
Distilled water to	6 liters

To prepare I. P. B. B. No. 3: Weigh into 6 liter volumetric flask, Na_2HPO_4, Na citrate, and KCl as given in formula. Dissolve in about 5 liters of distilled water. Weigh $CaCl_2$ (0.029 gm.) into clean container, dissolve and wash contents into 6 liter volumetric flask. Autoclave salt mixture. Weigh with sterile precautions 152.0 gm. glycerol into clean sterile bottle. Wash contents with sterile distilled water into 6 liter volumetric, cooled to room temperature. Make up to mark with sterile distilled water at room temperature. This procedure was followed for convenience despite the small error introduced by autoclaving the volumetric flask.

Electric Transport of Fluid Across Serous Membranes.

Theoretical.—The volume of fluid transported by an electric current in unit time across a simple membrane, traversed by channels whose walls are of uniform material, is defined by the equation[8]

$$\Phi = \frac{K \cdot I \cdot \sigma \cdot \zeta}{4\pi \cdot \eta} \qquad (1)$$

in which Φ = volume of fluid transported in unit time across the membrane, K = dielectric constant of the solution in the membrane pores, σ = specific resistance of the solution in the membrane pores, η =

[8] von Smoluchowski, M., in Graetz, L., Handbuch der Elecktrizität und des Magnetismus., Leipzig, 1912, ii, 380.

viscosity coefficient of the solution in the membrane pores, I = electric current traversing the membrane pores, ζ = electrokinetic P.D.[9] between pore walls and solution in the pores (at the isoelectric point $\zeta = 0$).

The direction and magnitude of the liquid flow with reference to the electric current, other factors remaining constant, depend upon the values of ζ. These in turn are determined by the materials with which the membrane channels are coated and upon the nature and concentration of the ions, chiefly the H ions, in contact with the membrane materials. Thus if the channel walls are of amphoteric substance the membrane will have a hydrion reversal point, on the alkaline side of which streaming is toward the cathode and on the acid side, toward the anode.

Animal membranes of the type studied in the experiments here reported are not simple but heterogeneous in composition and complex in arrangement of parts. It does not seem theoretically possible to define the volume of liquid flow through such a membrane. Nevertheless it is clear that each minute part of the membrane must influence (except at its isoelectric point) the drive of the liquid toward anode or cathode. Some concentration of hydrogen ions in the perfusing fluid should be discoverable, at which the fluid currents of various values, in different directions, flowing through the minute surface elements of the membrane, together with the various circulatory currents within the membrane, so neutralized each other that no accumulation of fluid on either side of the membrane results.

The hydrogen ion reversal point, or more briefly, the *reversal point* of a complex, amphoteric membrane may be defined empirically, then, as *that concentration of H ions in a given solution within the pores of the membrane, traversed by an electric current, at which the total electroendosmotic current through the membrane is 0.*

The need for specifying the composition of the solution bathing the membrane follows from consideration of recent work by Michaelis and Rona. They have shown that the dissociation state of an acid, a base, or an ampholyte, is influenced by the presence of any ions, if there is affinity for them, through salt formation.[10] Citrate ion dis-

[9] Freundlich, H., and Gyemant, A., *Z. physik. Chem.*, 1922, c, 182.

[10] Michaelis, L., and Rona, P., *Biochem. Z.*, 1919, xciv, 225. Michaelis, L., *Biochem. Z.*, 1920, ciii, 225; cvi, 83.

places rather strongly to the acid side. It will be shown in a later communication that the polyvalent anions present in the buffers used in the present experiments displaced the H ion reversal point toward the acid side by a considerable fraction of a pH unit.

It seems clear that if such a reversal point is found experimentally the corresponding H ion concentration must be somewhere between the extreme values which correspond to the isoelectric points of the various membrane substances involved.

It will be suggested below that the histological structure of the lean membranes, together with the position and sharpness of the H ion reversal points found for them seem to indicate that their electrokinetic P.D. are primarily referable to the dissociation state of their proteins. Little difference was found in the values of the reversal points for the fat pericardia tested in the early hours post mortem as compared with the lean pericardia. It seems necessary to conclude either that the surfaces of the fat cells introduce no driving force of their own or that their driving force, and hence electrokinetic P.D., must vary in much the same way with respect to hydrogen ion concentration as do the substances of the lean membrane.

Knowledge of the electrokinetic behavior of the non-protein substances of cells is too meager to justify positive conclusions as to which possibility is correct. Ellis[11] found, nevertheless, that hydrocarbon oils suspended in water migrated to the anode. Their negative charge was not reversed in HCl even up to N/10. Heesch[12] also showed anodal migration for cholesterol and lecithin. Probability seems, therefore, to favor the supposition that the behavior of the electrokinetic P.D. of the fat cell surfaces is much like that of the substances of the lean membranes. These experiments afford presumptive evidence, then, that proteins are an important or predominant element in the composition of the fat cell surfaces.

EXPERIMENTAL.

The experimental procedure was simple. Morphine sulfate (1.5 cc. of a 1 per cent solution) was usually injected subcutaneously into the dogs and cats from ½ to 2½ hours before anesthesia. Rabbits were not morphinized. The operation

[11] Ellis, R., *Z. physik. Chem.*, 1912, lxxviii, 321.

[12] Heesch, K., *Arch. ges. Physiol.*, 1921, cxc, 198.

was always performed under ether. A mid-line abdominal incision was made, the omentum raised, and a loop of intestine delivered. A clear intervascular window of mesentery was then washed with the buffer to be used and applied to the electrode vessel as described above (Figs. 1 and 2).[13] Buffer was introduced into the electrode vessel through the vertical rubber tube and the tube clamped. The membrane over the mouth of the electrode vessel and the agar electrode were then immersed in buffer in a crystallizing dish, the level of the meniscus read, and the circuit closed. The current was allowed to flow for 2 minutes with inner electrode cathode, and the meniscus read; a pole-charging switch was thrown, and the current was allowed to flow 2 minutes with inner electrode anode, or four alternate 1 minute runs were made. The algebraic sum of rise to the cathode and fall from the anode during the 4 minutes of current flow was taken as the movement of the meniscus for the total period. It can readily be shown that if a small constant leakage of buffer through the membrane due to hydrostatic pressure was occurring, this was practically cancelled out by the method adopted. Continued tapping on the electrode vessel was of assistance in preventing the accumulation of bubbles from the platinum electrode from interfering with the reading.

In the early experiments, dilute blood of the experimental animal and McIlvaine's buffer near pH = 5.7 were used with as many regions of the serosæ as possible. The mesocolon and mesocecum, the mesentery of the small intestines, the omentum, pericardium, and various parts of the parietal pleura and the pleura between the lobes of the lungs were tested. In all, liquid flow was found to be uniformly toward the cathode. The various membranes were tested also with buffers of pH near 3.3., and flow was regularly toward the anode. It proved possible in these experiments to test the pericardium and pleura while the animal's heart was still beating, though respiration had stopped.

Those sites which showed themselves in these experiments sufficiently accessible and strong were adopted for routine use. For quantitative work it was necessary also to take more efficient precautions against contamination of the buffers with blood than was possible with the pericardium or pleura of the dying animal. The animals were thereafter bled to death, therefore, by opening the femoral arteries, and occasionally also the aorta. The membranes could then be washed practically free of blood and tissue juice before determination of the reversal points.

Estimation of the reversal points of the thicker membranes, particularly those containing fat deposit, was complicated by the difficulty of washing one buffer from the membrane pores before using another. This may be illustrated by Fig. 3, Experiment 50. 4 minute runs with the same site of the membrane bathed in buffers of all pH values indicated by the abscissæ were made in the order of the numbered points. The ordinates indicate the transport of fluid toward cathode

[13] Electrode vessel and agar electrode were, of course, supported by clamps not shown in Fig. 1.

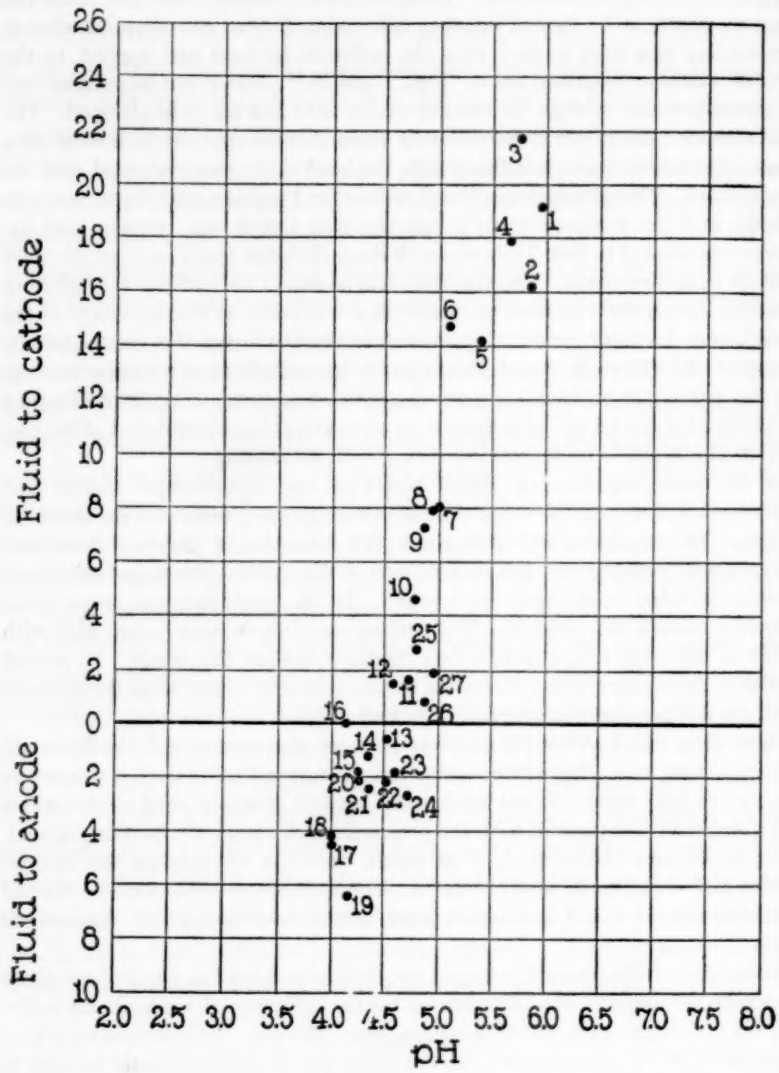


FIG. 3. Experiment 50. Human pericardium. I. P. B. B. No. 2. Abscissæ indicate pH of buffers bathing outside of membrane. Ordinates represent volume of liquid transported in c.mm. per minute per milliampere of current. Runs numbered in the order made.

or anode in cubic millimeters per minute per milliampere of current. Successive runs in this experiment were made with buffers of successively lower pH, from pH = 5.98 to pH = 4.04. The resulting curve crossed the base line at a pH of about 4.55. Buffers in the order of increasing pH were then used and a second point of intersection with the base line was found at pH = 4.8. The "corrected reversal point" is the estimated point at which, in the experimenter's judgment, the curve would have crossed the base line could this lag in filling the membrane pores with the desired buffer have been entirely eliminated.

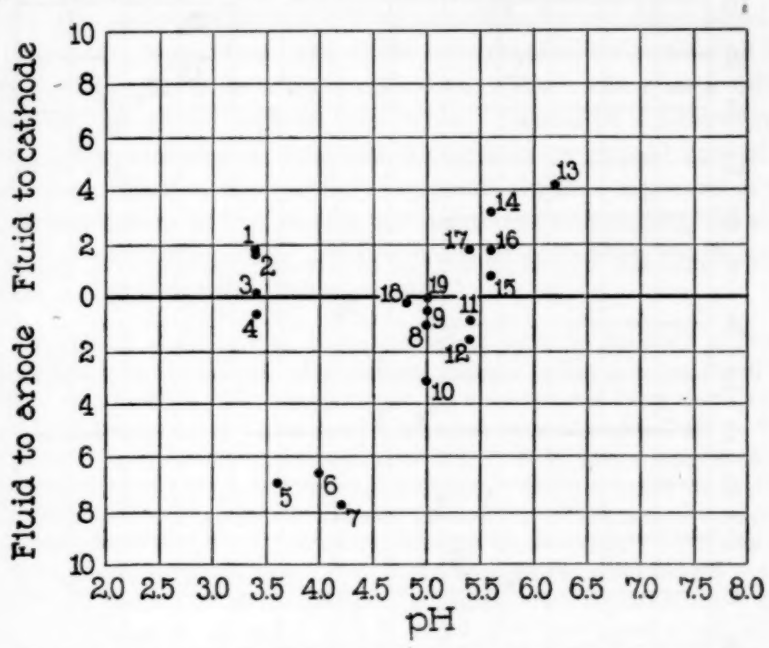


FIG. 4. Experiment 57. Dog pericardium, fat. I. P. B. B. No. 3. Illustrates estimation of reversal point. Coordinates as in Fig. 3.

With many membranes, particularly the fat ones, the discrepancy between the apparent reversal points proved to be much greater than in Experiment 50, so that these served only to define the lower and upper limits, respectively, between which the true reversal point lay. Another procedure, therefore, had to be adopted for estimation of the corrected reversal point. This is illustrated in Fig. 4. Buffer in the electrode vessel and crystallizing dish was changed in the middle and at the end of the 4 minute period. Successive runs narrowed down the limits within which the true reversal point lay until the point was at least approximated in this case by Run 19. In later experiments, when the approximate position of the reversal point had become known, a few buffers of about the critical reaction were

used for a large number of runs. The reversal point could thus be determined objectively within 0.2 pH unit and by interpolation could be estimated considerably more closely.

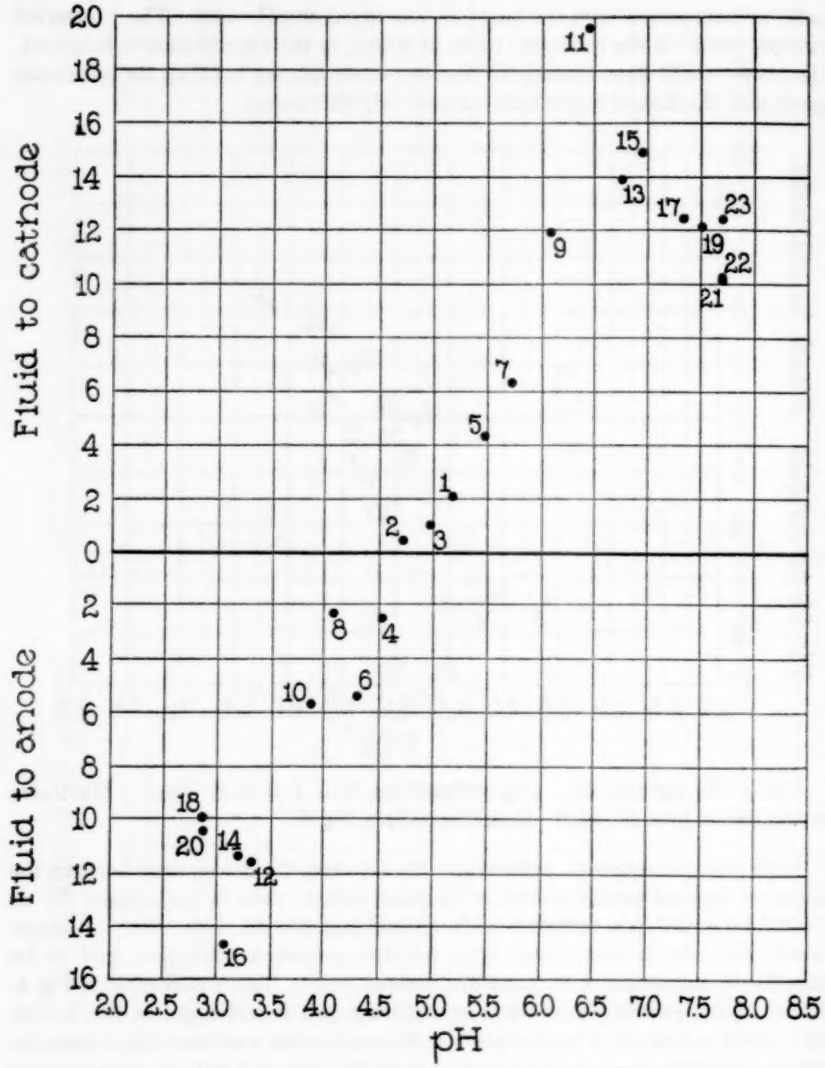


FIG. 5. Experiment 40. Rabbit pericardium, lean. Dilute McIlvaine's buffer. Illustrates reversibility of membrane. Runs numbered in the order made.

The ready *reversibility* of the membranes proved to be a striking attribute. Fig. 5, Experiment 40 may serve to illustrate this point. From points 3 to 21, inclusive, each run was in a direction opposite to its predecessor. These 18 reversals of the charge on the membrane were made in a total time of 138 minutes, and without greatly distorting the shape of the curve. Repeated reversals of the direction of flow across the same sites of the mesenteries of living dogs were also made by alternately using an acid buffer and dilute blood.

Attempts to gain some idea of the time required for reversal of the tissues were made on other membranes. Observations on a rabbit pericardium afford the best illustration. Successive 2 minute runs were made attesting six reversals of charge in an elapsed time of 20 minutes. Most of this certainly represents the time expended in the runs themselves and in washing the membrane and refilling the electrode vessel between runs. At best it sets an exceedingly rough upper limit on the time required for reversal.

Comparison of the reversal points determined with the *hypotonic McIlvaine buffer* and with the *isotonic, physiologically balanced buffers* does not reveal unequivocal differences. Ten experiments on the mesenteries of living animals with dilute McIlvaine's buffer gave a mean apparent reversal point of pH = 4.42. Eight similar experiments on mesentery with I. P. B. B. gave a mean apparent reversal point of pH = 4.43, a striking agreement. In the experiments on these delicate membranes, a new site was used for each run. The mean reversal point of three experiments on cat pleura with McIlvaine's buffer was pH = 4.37; for two experiments on cat pleura with I. P. B. B. the mean reversal point was pH = 4.25. Three dog pleuræ with McIlvaine's gave an average reversal point of pH = 4.96, and a single dog pleura with I. P. B. B. No. 3, a reversal point of pH = 5.0. The mean reversal point of fourteen pericardium experiments with McIlvaine's was 5.18 ($\pm$ 0.026, the probable error of the mean); of 5 pericardium experiments with I. P. B. B. was 5.04 $\pm$ 0.081. In the pericardium and pleura experiments one site was used for the whole reversal point determination. The membranes were thus exposed to the action of the hypotonic unbalanced buffer long enough for considerable swelling to occur. The slight differences between the mean reversal points of the pericardia may have been purely fortuitous or may have been due to some effect upon the membrane by the McIlvaine buffer. At all events the lack of a more definite difference of reversal point determined with the two types of buffer is the point of interest here.

Comparison of the reversal points of *fat* and *lean* pericardia has shown only small differences. The mean reversal point for eleven lean pericardium experiments was pH = 5.18 $\pm$ 0.046; for eight fat pericardium experiments, the mean

reversal point was 5.08 ± 0.022. Consideration of the experiments of Table I indicates, however, that a slightly greater concentration of hydrogen ions may be required to reverse the fat than the lean region of the same pericardium. For

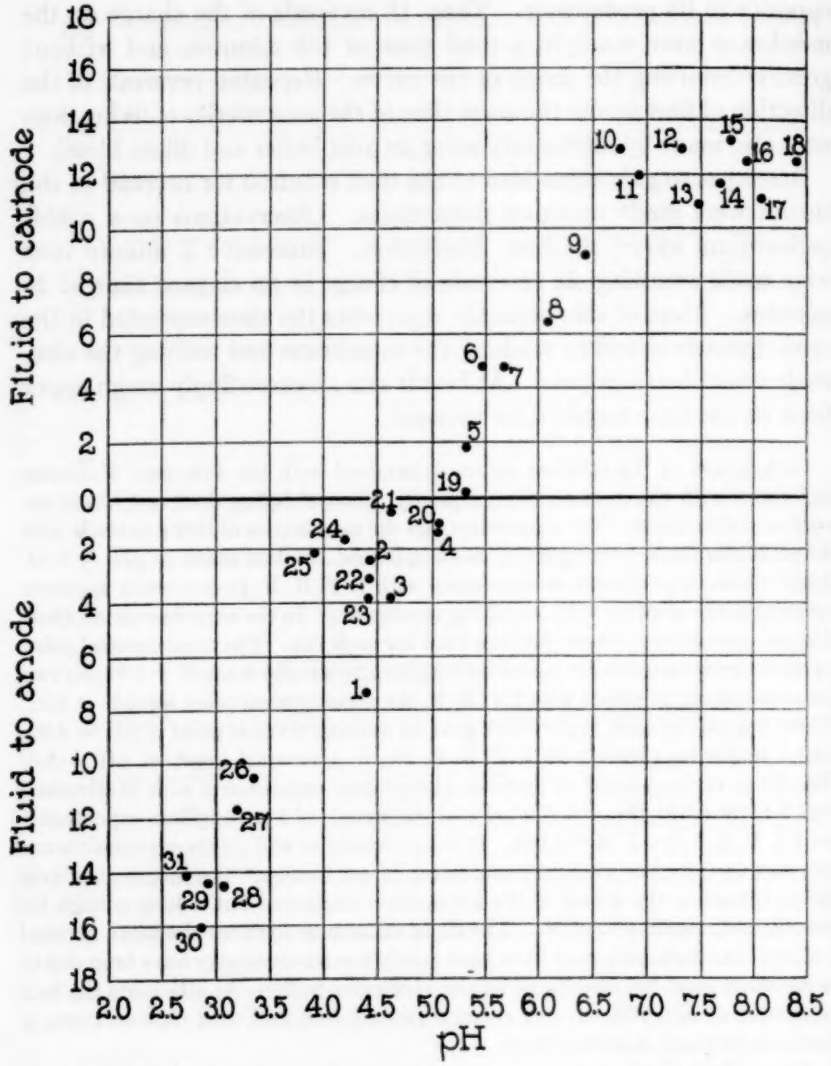


FIG. 6. Experiment 41. Cat pericardium, lean. Dilute McIlvaine's buffer. 1–18, first experimental site; 19–31, second site.

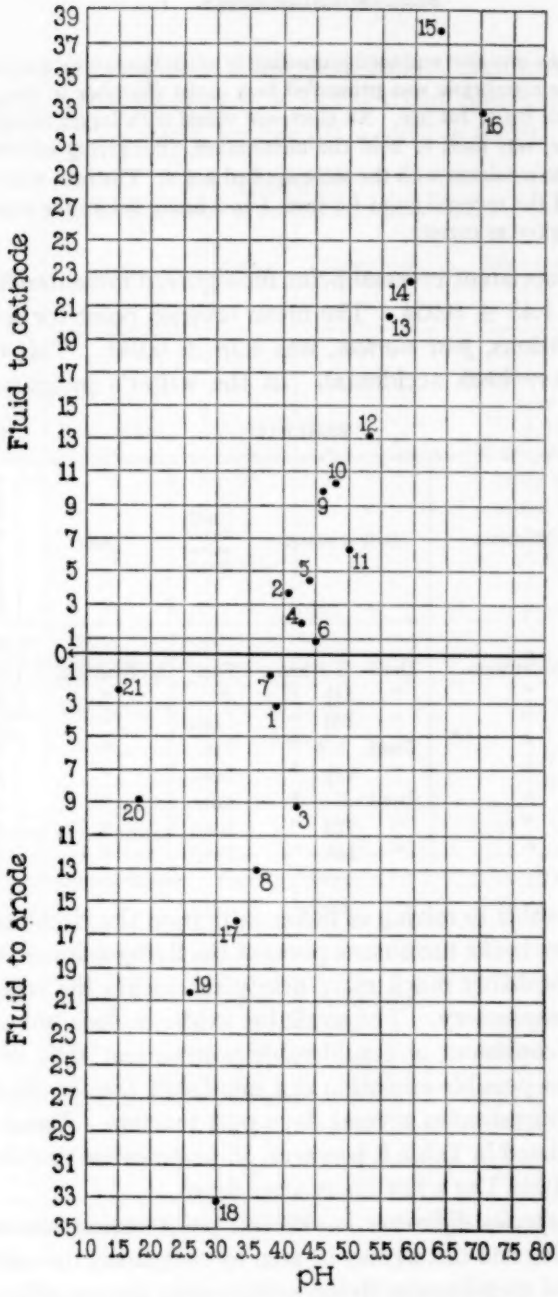


FIG. 7. Experiment 45. Mesentery of living cat. I. P. B. B. No. 1.

these estimations one site was used immediately after the sacrifice of the animal. The rest of the membrane was preserved in a moist chamber in the ice box at about 4°C. until ready for use. An electrode vessel with larger mouth, 14 mm. inside diameter, was used to hold the membranes, thus giving somewhat larger excursions of the meniscus with the same applied E.M.F. The runs were continued in the region of the reversal point for from 1 to 3 hours before the reversal point found was accepted as correct.

The mean apparent reversal point for eighteen mesenteries of *living animals* was 4.43 ± 0.034. The mean reversal point for seven peritoneal estimations, *post mortem*, was 4.78 ± 0.044. This difference could not have been accidental. In the writer's judgment it was

TABLE I.

Experiment No	Dog.	Membrane.	State of animal.	Condition of membrane.	Buffer.	Duration of experiment.	Reversal point. Approximate pH.
						min.	
65	A.	Pericardium.	Dead 3+ hrs.	Fat.	McIlvaine's.	163	5.15
66	"	"	" 52½ "	"	"	181	5.1
69	"	"	" 190 "	Lean.	"	87	5.3
67	B.	"	Dead 2+ "	Fat.	"	157	5.05
70	"	"	" 93½ "	Lean.	"	111	5.35
68	C.	"	Dead 2+ "	Fat.	"	149	5.2
71	"	"	" 119 "	Lean.	"	163	5.2
72	"	"	" 146 "	Fat.	"	168	5.0

probably referable to mixing of buffer salts from the circulating blood with the buffer in the membrane pores of the living animals; the value pH = 4.78 probably much more nearly represents the true reversal point of the mesentery. The available evidence does not appear to indicate any consistent or considerable postmortem shift of reversal point, with the possible exception of a small shift toward the acid side, with the fat membranes several days post mortem. Except for the experiments listed in Table I, however, all postmortem determinations were made within 1 or a few hours after death.

A definite *species difference* in reversal point values seems to exist between the dog and cat, as may be seen by comparing the mean values for the several membranes: living peritoneum, for cat pH = 4.36 ±

0.029, for dog pH = 4.49 ± 0.064; pericardium, post mortem, for cat pH = 4.99 ± 0.096, for dog pH = 5.19 ± 0.026; pleura, post mortem, for cat pH = 4.32 ± 0.058, for dog pH = 4.97 ± 0.019.

TABLE II.

No. of experiments.	Species.	Membrane.	State of animal.	Condition of membrane.	Mean reversal point.	Range of variation.
					pH	*pH*
					Apparent reversal point.	
2	Rabbit.	Peritoneum.	Living.	Lean.	4.37	0.1
7	Cat.	"	"	"	4.36 ± 0.029*	0.3
9	Dog.	"	"	"	4.49 ± 0.064	0.9
					Corrected reversal point.	
4	Cat.	"	Dead.	"	4.80	0.3
2	Dog.	"	"	"	4.62	0.15
1	"	"	"	Fat.	5.0	
Average, dog peritoneum, dead					4.75	0.45
2	Human.	Pericardium.	Dead.	Lean.	4.98	0.6
1	Rabbit.	"	"	"	5.1	
2	Cat.	"	"	"	4.9	0.6
2	"	"	"	Fat.	5.07	0.25
Average, all cat pericardia					4.99 ± 0.096	0.6
8	Dog.	Pericardium.	Dead.	Lean.	5.26 ± 0.032	0.45
6	"	"	"	Fat.	5.08 ± 0.021	0.2
Average, all dog pericardia					5.19 ± 0.026	0.45
1	Rabbit.	Pleura.	Dead.	Partly lean.	4.85	
5	Cat.	"	"	Lean.	4.32 ± 0.058	0.5
4	Dog.	"	"	"	4.97 ± 0.019	0.1
Average, 18 living peritoneum experiments					4.43 ± 0.034	0.92
" 7 dead		"	"		4.78 ± 0.044	0.47
" 11 lean pericardium			"		5.18 ± 0.046	0.85
" 8 fat		"	"		5.08 ± 0.022	0.25

* The precision measure given, ±0.029 etc., is in each case the probable error of the mean.

The data of several experiments are shown graphically for illustration. The reversal point data are summarized in Table II.

DISCUSSION.

Electroendosmotic experiments with organized tissues have been reported previously by but few authors.

Girard [14] pointed out in 1910 that some of the values of apparent osmotic pressure determined by Graham with pig bladder osmometers were doubtless due to electroendosmosis superposed on osmosis in the sense of van't Hoff. Bartell and his collaborators[15] have published very important work on electroendosmotic effects in gold-beaters skin, parchment paper, and other membranes as the cause of anomalous osmosis. Girard and Morax[16] secured electroendosmotic transport across the corneas of rabbits *in situ;* streaming was to the cathode but could be reversed when the eyeball was bathed in $BaCl_2$. Girard and his coworkers have driven solutions electroendosmotically also into cancer[17] and liver tissue.[18] Stern[19] found cathodal streaming through willow twigs and sections of potato soaked with conductivity water. Heesch [12] has reported the reversibility of a variety of cells and cell components by lanthanum salts and by salts of histone, clupeine, and of several basic dyes. Cataphoresis was used. Pig bladder and frog muscle were found by electroendosmosis to be reversible by the same agents.

Regarding the bearing of the experiments here reported on the important question of the chemical composition of the water-pervious channels through the membranes, the ready reversibility of the membranes with hydrogen ions seems to the writer to constitute strong evidence that the charging of the membrane ultimately depends in greater part, at least, upon the dissociation state of certain amphoteric components. The proteins are well known to be similarly sharply reversible in their dissociation state and the reversal points of the membranes lie in the characteristic protein isoelectric zone (see Table III). Indeed, the behavior of the membranes affords rather striking analogy to the results of some of the experiments of Loeb with protein-coated collodion membranes.[20]

[14] Girard, P., *Compt. rend. Acad.*, 1910, cli, 99.

[15] Bartell, F. E., and Hocker, C. D., *J. Am. Chem. Soc.*, 1916, xxxviii, 1029, 1038. Bartell, F. E., and Madison, O. E., *J. Phys. Chem.*, 1920, xxiv, 444, 593. Bartell, F. E., and Sims, L. B., *J. Am. Chem. Soc.*, 1922, xliv, 289. Bartell, F. E., and Carpenter, D. C., *J. Phys. Chem.*, 1923, xxvii, 101, 252, 346.

[16] Girard, P., and Morax, V., *Compt. rend. Acad.*, 1920, clxx, 821.

[17] Roffo, A.-H., and Girard, P., *Compt. rend. Acad.*, 1920, clxxi, 273.

[18] Fauré-Fremiet, E., and Girard, P., *Compt. rend. Soc. biol.*, 1921, lxxxv, 1140.

[19] Stern, K., *Z. Bot.*, 1919, xi, 561.

[20] Loeb, J., Proteins and the theory of colloidal behavior, New York and London, 1922.

On the other hand, the only known ampholytic lipoids, the phosphatides,[21] have a broad isoelectric zone (see Table III) in which the whole molecules are electrically neutral. The possibility, indeed probability, that lipoids, when oriented in interfaces, may present charged surfaces or may attain a charge through adsorption, of course, remains. There is no reason, however, to expect a sharp reversal point such as has been found in the present experiments as a consequence of the presence of lipoids, although a reversal point due to protein in the presence of a certain amount of lipoid is, of course, possible.

Behavior of the membranes in a way suggestive of protein composition is not surprising with regard to such membranes as the lean pericardium or pleura in which the major bulk of the tissue is intercellular fibrous material of collagen and elastin. It would seem that in the fat membranes, however, a large part of the course of the perfusing fluid must be between the thin protoplasmic shells of the fat cells. The conclusion seems warranted that the surfaces of these cells probably contain protein in a predominant or at least an important amount. (See page 396.)

It may be argued that exposing the serosæ and treating them with fluids at reactions so far removed from the normal reaction of the blood may alter them from their normal condition. While this possibility cannot be denied, nevertheless the force of the objection is considerably abated by the following considerations. No considerable differences were detected between the behavior of the membranes when bathed in isotonic, physiologically balanced buffers and such grossly injurious mixtures as hypotonic solutions of pure sodium salts. Significant differences were not detected between membranes in the first as compared with subsequent hours post mortem with one possible exception. Only small differences in direction and amount of liquid transport appeared as a result of varying the order in which the membranes were exposed to the acid and alkaline buffers.

A list of published isoelectric points of biological substances is appended for reference. These are of very uneven value. Certain of the figures have been determined with precision, *e.g.* Sörensen (25); others are at best approximations; the values of Michaelis and Davidsohn for pepsin and trypsin are probably in error altogether. Critical appraisal is not attempted here; the originals should be consulted.

[21] The writer is indebted to Dr. P. A. Levene for this information.

TABLE III.

Isoelectric Points of Substances of Biological Interest as Previously Recorded.*

Reference.	Substance.	H ion concentration of isoelectric point, determined		
		By cataphoresis. (H^+)	By flocculation optimum. (H^+)	pH (approximate).
Michaelis and Davidsohn (1)	Genuine serum albumin.	2×10^{-5}	—	4.7
" " " (1)	Denatured " "	4×10^{-6}	4×10^{-6}	5.4
Rona and Michaelis (2)	Serum globulin.	Approx. 4×10^{-6}	Approx. 3.6×10^{-6}	5.4
Michaelis and Davidsohn (3)	Typhoid agglutinin.	1.5×10^{-6}	" 3.6×10^{-6}	5.4
" " Pechstein (4)	Casein (cow).	2.5×10^{-5}	2.4×10^{-5}	4.6
Cohn, Gross, and Johnson (5)	Carrot protein.	Approx. 10^{-4}	Approx. 10^{-4}	4.0
Michaelis and Mendelssohn (6)	Edestin.	—	" 2.5×10^{-6}	5.6
" " Grineff (7) / Loeb (8)	Gelatin.	Approx. 2.5×10^{-5}	2.0×10^{-5}	4.7
Rona and Michaelis (2)	Gliadin.	" 5.9×10^{-10}	Approx. 5.9×10^{-10}	9.2
Cohn, Gross, and Johnson (5)	Tomato protein.	" 10^{-5}	" 10^{-5}	5.0
Cohn (9)	Tuberin.	" 2.5×10^{-6}	—	5.6
Fodor (10)	Yeast extract protein (globulin).	—	Approx. 3.4×10^{-5}	4.5
Michaelis and Davidsohn (11)	Nucleic acid from yeast.	—	" 2×10^{-1}	0.7
" " Rona (12)	Nucleoprotein from yeast; contains or is maltase.	—	" 6×10^{-5}	4.2
" " Bien (13)	Reduced hemoglobin.	1.7×10^{-7}	—	6.8
" " " (13)	Oxyhemoglobin.	1.7×10^{-7}	—	6.8
" " " (13)	CO-hemoglobin.	1.7×10^{-7}	—	6.8
" " Takahashi (14)	Stroma globulins of red blood cells.	—	1×10^{-5}	5.0
Kugelmass (15)	Fibrin.	Staining and swelling methods 6.3×10^{-8}		7.2
" (15)	Fibrinogen.	" " turbidity maximum 1×10^{-8}		8.0
Quagliariello (16)	Hemocyanine (octopus).		2.1×10^{-5}	4.7
Michaelis and Davidsohn (3)	Agglutinable substance of *B. typhosus*.	—	4×10^{-6}	4.4
Michaelis and Pechstein (17)	Catalase of liver.	Approx. 4.3×10^{-6}	—	5.4
" " " (18)	Salivary diastase.	Between 10^{-5} and 10^{-6}	—	5.0–6.0
" " Davidsohn (19)	Pepsin.	Approx. 5.5×10^{-3}	—	2.3
Ringer (20)	" Wanders always to			

Quaglariello (16)	Hemocyanine (octopus).			
Michaelis and Davidsohn (3)	Agglutinable substance of *B. typhosus*.	—	4×10^{-6}	4.4
Michaelis and Pechstein (17)	Catalase of liver.	Approx. 4.3×10^{-4}	—	5.4
" " " (18)	Salivary diastase.	Between 10^{-4} and 10^{-6}	—	5.0–6.0
" " Davidsohn (19)	Pepsin.	Approx. 5.5×10^{-3}	—	2.3
Ringer (20)	" Wanders always to anode.	—	—	—
Michaelis and Davidsohn (21)	α-nucleoprotein of pancreas, "trypsin."	Approx. 1.35×10^{-4}	Approx. 2.6×10^{-4}	3.7
" " " (21)	β-nucleoprotein of pancreas.	—	1.2×10^{-3}	2.9
Northrop (22)	Trypsin.	Method depending on Donnan equilibrium 6.3×10^{-11}		10.2
Collip (23)	Myosin.	—	$5.0 \times 10^{-7} - 2.5 \times 10^{-7}$	6.3–6.6
Somogyi, Doisy, and Shaffer (24)	Insulin.	—	10^{-5}	5.0
Sörensen (25)	Egg albumin (hen).	Titration curve method 1.57×10^{-5}		4.8
Thomas and Kelly (26)	Hide powder.	Methods of staining and of swelling minimum approx. 10^{-5}		5.0
Coulter (27)	Red blood cells.	2.5×10^{-5}	—	4.6
Winslow, Falk, and Caulfield (28)	Bacterial "	Approx. 10^{-3}	—	3.0
" and Shaughnessy (29)	" " second isopotential point.	" 3.2×10^{-14}	—	13.5
Robbins (30)	Potato tuber tissue.	Staining and water absorption methods approx. 10^{-6}		6.0
" (31)	Mycelium of *Fusarium lycopersici*.	Staining methods approx. 3.2×10^{-6}		5.5
" (31)	Mycelium of *Rhizopus nigricans*.	" and growth minimum methods approx. 10^{-5}		5.0
Scudder (32)	Isoelectric points of amino-acids can be computed from dissociation constants.			
Levene, Rolf, and Simms (33)	Lysolecithin (titration method), isoelectric range, pH = 2.75 – 9.90.			
" " " " (33)	Lysocephalin (" "), " " pH = 5.5 – 7.5.			

* The term isoelectric was originally applied by Hardy (34) to condition in which protein particles showed no migration in an electric current, *i.e.* in which their electrokinetic P.D. was 0. Michaelis subsequently showed that isoelectric point of a pure ampholyte was defined by equation $I.P. = \sqrt{\frac{Ka}{Kb} Kw}$ Ka is acid and Kb basic dissociation constant of the ampholyte, and Kw the dissociation constant of water. In the second edition of Michaelis' monograph (35) isoelectric point is used both in Hardy's empirical sense and in the sense of the special, mathematically defined case. In this table, empirical sense is intended for cells and tissues, whose electrokinetic P.D. cannot be assumed referable to a simple source, and the sense of special case is intended for amino acids and pure proteins.

SUMMARY.

It is shown that when a mammalian serous membrane bathed in dilute buffer is traversed by an electric current, liquid is caused to stream through the membrane toward the cathode when the pH value of the buffer is on the alkaline side of a certain critical hydrogen ion concentration. Streaming is toward the anode on the acid side of the reversal point. Simple means for studying this electroendosmosis quantitatively are described. The mean values of the reversal points in all cases studied with the present buffers lie between pH = 4.3 and 5.3.

The membranes studied have been the mesentery of the living and dead animal, and the parietal pericardium and pleura, post mortem. The membranes of dogs, cats, rabbits, and two human pericardia have been studied. All these membranes are essentially sheets of connective tissue, bearing blood vessels, lymphatics, and nerves, and in some instances fat cells, and lined on each surface by a single layer of pavement mesothelial cells. Intercellular fibers form the major bulk of the lean membranes. These are predominantly collagenous except in the pleural region used, in which elastin fibers are present in large proportion.

By using buffers alternately more acid or more alkaline than the reversal point, the direction of liquid flow across any given membrane site could be reversed an indefinite number of times. The time interval required is only that taken by the requisite manipulation in changing buffers and making the runs.

The mean values of the reversal points for the several membranes when bathed in hypotonic, unbalanced buffer and in isotonic, physiologically balanced buffers showed only small and inconstant differences. The fat and lean pericardia similarly showed small difference in the positions of the mean reversal points.

The apparent reversal points for the mesenteries of living animals proved to be lower than those for the mesenteries post mortem. This low value in the animals with functioning circulation is interpreted as essentially due to admixture with the buffers of buffer salts from the blood. Clear differences between the reversal points of the membranes in the first compared with later hours or days post mortem were

not detected, with the possible exception of a small shift toward the acid range of the fat pericardium reversal points estimated several days post mortem. The reversal points with cat membranes were somewhat lower (more acid) than those of the dog.

The approximate mean reversal points found with the citrate-phosphate buffers used were as follows: For mesenteries of living animals pH = 4.4; for mesenteries, post mortem, pH = 4.8; for cat pleuræ, post mortem, pH = 4.3; for dog pleuræ, post mortem, pH = 5.0; for lean and fat pericardia, post mortem, pH = 5.1. The mean reversal point of the two human pericardia was about pH = 5.0. Reversal points determined with buffers containing only monovalent anions are somewhat higher (less acid), as will be shown later.

The bearing of these data on the question of the chemical composition of the surfaces of the fat cells of the serous membranes is discussed. Briefly, it is believed that proteins are probably present in important amount in these cell surfaces.

The writer wishes to express his indebtedness to Dr. Harry Clark for invaluable aid with the theoretical considerations involved in this paper.

Protocols.

Experiment 50.—(Fig. 3.) Human pericardium. I. P. B. B. No. 2. Adult male, about 40 yrs. Died 9:45 a.m., Mar. 25, 1924. Lobar pneumonia, positive blood culture. 4 minute runs on same site of pericardium from 11:53 a.m. to 4:01 p.m. For structure of membrane see Fig. 4, Plate 2.

Experiment 57.—(Fig. 4.) Dog pericardium. Male. I. P. B. B. No. 3. Membrane contained fat deposit in experimental site. Animal succumbed to bleeding 1:06 p.m. 4 minute runs on same site from 1:49 p.m. to about 5:40 p.m.

Experiment 40.—(Fig. 5.) Rabbit pericardium, lean. Female. Dilute McIlvaine's buffer. Animal died of bleeding 12:59 p.m. 4 minute runs on same site from 1:27 p.m. to 4:35 p.m.

Experiment 41.—(Fig. 6.) Cat pericardium, lean. Male. Dilute McIlvaine's buffer. Animal died of bleeding 1:07 p.m. 4 minute runs on first site from 1:38 p.m. to 3:51 p.m.; similar runs on second site from 4:13 p.m. to 5:53 p.m.

Experiment 45.—(Fig. 7.) Cat mesentery, lean. Male. I. P. B. B. No. 1. 1.5 cc. of 1 per cent morphine sulfate subcutaneously, 11:45 a.m. Ether anesthesia begun 1:25 p.m. Mid-line abdominal incision. 4 minute runs, each on a different site of mesentery, from 1:45 p.m. to 4:54 p.m. Perivascular fat deposit encroached considerably on intervascular areas.

BIBLIOGRAPHY TO TABLE III.

1. Michaelis, L., and Davidsohn, H., *Biochem. Z.*, 1911, xxxiii, 456.
2. Rona, P., and Michaelis, L., *Biochem. Z.*, 1910, xxviii, 193.
3. Michaelis, L., and Davidsohn, H., *Biochem. Z.*, 1912, xlvii, 59.
4. Michaelis, L., and Pechstein, H., *Biochem. Z.*, 1912, xlvii, 260.
5. Cohn, E. J., Gross, J., and Johnson, O. C., *J. Gen. Physiol.*, 1919–20, ii, 145.
6. Michaelis, L., and Mendelssohn, A., *Biochem. Z.*, 1914, lxv, 3.
7. Michaelis, L., and Grineff, W., *Biochem. Z.*, 1912, xli, 373.
8. Loeb, J., *J. Gen. Physiol.*, 1918–19, i, 241.
9. Cohn, E. J., (forthcoming publication).
10. Fodor, A., *Kolloid Z.*, 1920, xxvii, 58.
11. Michaelis, L., and Davidsohn, H., *Biochem. Z.*, 1912, xxxix, 496.
12. Michaelis, L., and Rona, P., *Biochem. Z.*, 1913, lvii, 81.
13. Michaelis, L., and Bien, Z., *Biochem. Z.*, 1914, lxvii, 198.
14. Michaelis, L., and Takahashi, D., *Biochem. Z.*, 1910, xxix, 439.
15. Kugelmass, I. N., *Arch. Int. Physiol.*, 1923, xxi, 150. (Discrepancies in article cited have been corrected by personal communication.)
16. Quagliariello, G., in Winterstein, H., Handbuch der vergleichenden Physiologie, Jena, 1922, i, pt. 1, 611.
17. Michaelis, L., and Pechstein, H., *Biochem. Z.*, 1913, liii, 335.
18. Michaelis, L., and Pechstein, H., *Biochem. Z.*, 1914, lix, 95.
19. Michaelis, L., and Davidsohn, H., *Biochem. Z.*, 1910, xxviii, 1.
20. Ringer, W. E., *Arch. néerl. Physiol.*, 1917–18, ii, 571.
21. Michaelis, L., and Davidsohn, H., *Biochem. Z.*, 1911, xxx, 481.
22. Northrop, J. H., *J. Gen. Physiol.*, 1923–24, vi, 337.
23. Collip, J. B., *J. Biol. Chem.*, 1922, l, p. xlv.
24. Somogyi, M., Doisy, E. A., and Shaffer, P. A., *J. Biol. Chem.*, 1924, lx, 34.
25. Sörensen, S. P. L., *Compt. rend. trav. Lab. Carlsberg*, 1915–17, xii, 149.
26. Thomas, A. W., and Kelly, M. W., *J. Am. Chem. Soc.*, 1922, xliv, 195.
27. Coulter, C. B., *J. Gen. Physiol.*, 1920–21, iii, 309.
28. Winslow, C.-E.A., Falk, I. S., and Caulfield, M. F., *J. Gen. Physiol.*, 1923–24, vi, 177. (Includes bibliography.)
29. Winslow, C.-E. A., and Shaughnessy, H. J., *J. Gen. Physiol.*, 1923–24, vi, 697.
30. Robbins, W. J., *Am. J. Bot.*, 1923, x, 412.
31. Robbins, W. J., *J. Gen. Physiol.*, 1923–24, vi, 259.
32. Scudder, H., The electrical conductivity and ionization constants of organic compounds, New York, 1914.
33. Levene, P. A., Rolf, I. P., and Simms, H. S., *J. Biol. Chem.*, 1923–24, lviii, 859.
34. Hardy, W. B., *J. Physiol.*, 1899, xxiv, 288; 1905–06, xxxiii, 257.
35. Michaelis, L., Die Wasserstoffionenkonzentration, Berlin, 2nd edition, 1922.

EXPLANATION OF PLATES.

PLATE 1.

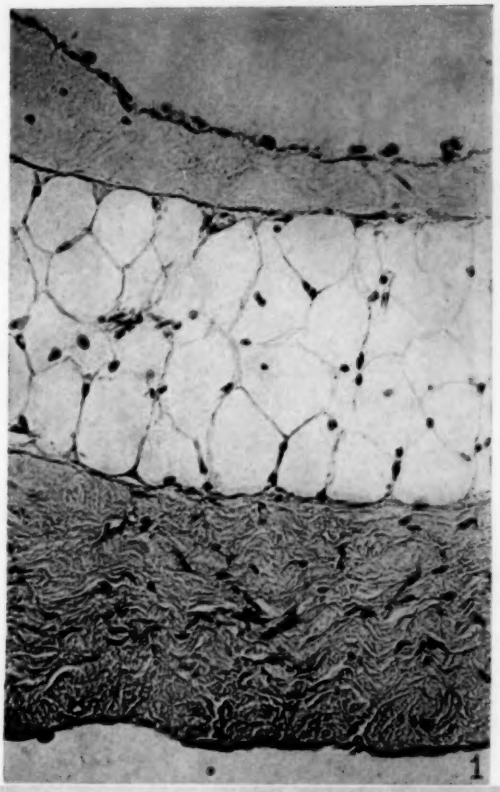
FIG. 1. Cat pericardium. Hematoxylin-eosin. The clear spaces in the fat layer are loci from which fat globules have been dissolved in preparation of the microscopic sections. × 260.

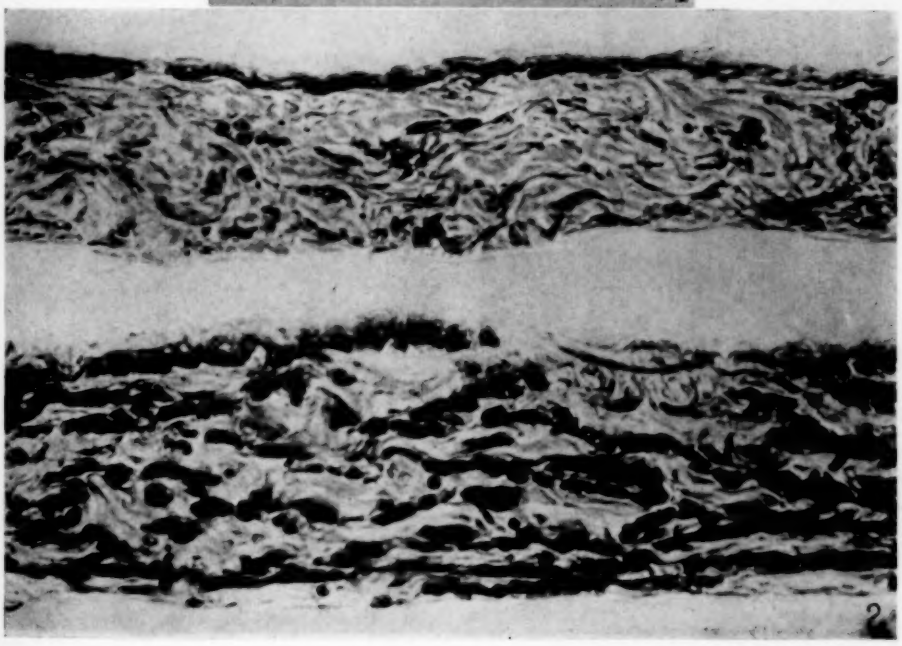
FIG. 2. Dog pleura. Weigert's elastic tissue stain. Two sections of pleura between pericardium and diaphragm. The elastin fibers appear black. × 510.

PLATE 2.

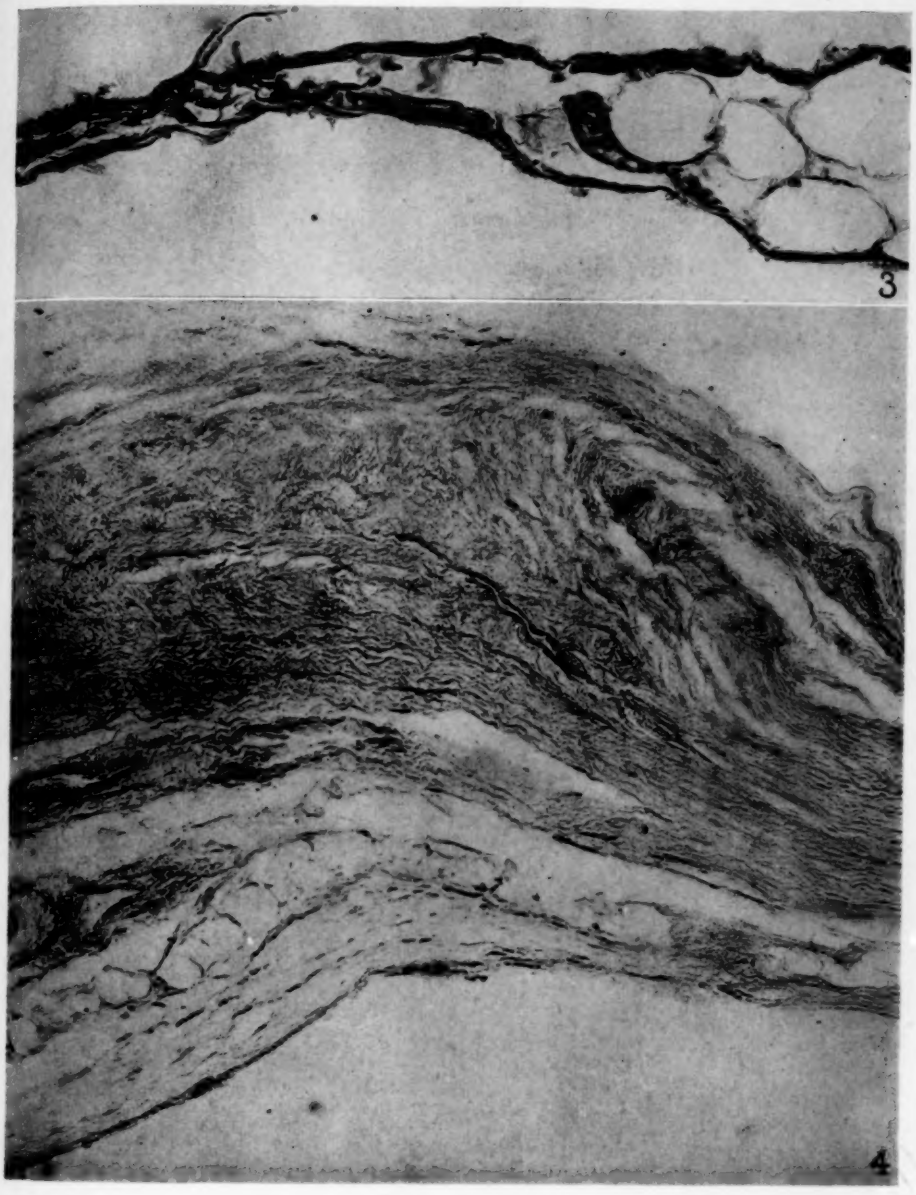
FIG. 3. Dog mesentery. Hematoxylin-eosin. × 260.

FIG. 4. Human pericardium. Weigert's elastic tissue stain. Section from site of Experiment 50. Only a few delicate elastin fibers imbedded in the white connective tissue. × 180.





(Mudd: Electroendosmosis. I.)



(Mudd: Electroendosmosis. I.)

THE LOCOMOTION OF LIMAX.

II. Vertical Ascension with Added Loads.

By W. J. CROZIER and H. FEDERIGHI.

(From the Zoological Laboratory, Rutgers University, New Brunswick, and the Carnegie Institution of Washington.)

(Accepted for publication, December 8, 1924.)

The inner mechanism of the pedal creeping organ of *Limax* responds so exactly to the action of temperature (Crozier and Pilz, 1923–24) that it has seemed desirable to study also the effect of varying the load to be moved by the pedal waves. It has been shown that the speed of vertically upward creeping, at constant temperature, is directly proportional to the frequency of pedal waves, to the advance produced by a single wave, and to the velocity with which the waves traverse the sole of the foot. When the temperature is varied, the frequency of the pedal waves required to lift the body vertically at given speed is proportional to the exponential of $\frac{1}{T}$, in which T is the absolute temperature.

With the technique described in the preceding paper (Crozier and Pilz, 1923–24) the efficiency of creeping was measured when the slug was forced to carry additional masses. Lead weights were attached by means of a thread to a bent pin inserted in the apex of the slug's dorsum. The pin, without weights, was present in control experiments, and was found to be without influence. The thread was long enough to prevent contact between the bit of lead and the body of the animal. In order to obviate the influence of phototropism the animals in some experiments were de-eyed.

The negative geotropism of *Limax* is as a rule precise. When the animal is placed at the bottom of a glass cylinder it begins to creep, encounters the wall, and orients upward. A number of instances appeared in which the orientation was not exactly vertical.

It was easy to show that if added traction be applied to the body muscles, by hanging a weight upon the hook inserted in the animal's back, orientation instantly became exactly upward. Thus a *Limax* with small lead mass attached by the thread might creep upward at an angle of about 45° upon the vertical wall of a cylinder, until the attached weight began to be lifted; the head end then immediately turned upward, and the vertical orientation was thereafter maintained. This probably proves that tension of the body muscles, produced by the pull of the animal's weight, is proprioceptively concerned in effecting geotropic orientation (as suggested for *Chiton*, which lacks statocysts (Arey and Crozier, 1919, *a*)). The experiment is of interest as demonstrating that the orientation of the anterior end is an active process.

As the loaded slug creeps vertically the speed of progression changes; it generally becomes at first faster, then slower; or it may change erratically. The effect is partly a function of the amount of additional mass. For our purpose the necessary information was obtained by measuring the time and counting the number of waves disappearing at the anterior end of the foot while moving upward over a known small distance (1.5 cm. or less) marked on the glass wall of the cylinder.

Fig. 1 demonstrates that while the velocity of spontaneous vertical ascension may be altered by added loads, the linear relation between this velocity (V) and the frequency (F) of the pedal waves is not affected; nor is the magnitude of the relation between the mean advance (A) due to a single wave and the speed (Fig. 2).

These results permit the conclusion that for equal rates of creeping the activity of the pedal organ is independent of the added loads. This is consistent with the view that the activities of the pedal organ are determined by its intrinsic neuromuscular structures; the nervous elements (nerve net) in the foot are only secondarily under the control of the central ganglia. In this respect the foot of *Limax* is comparable to the gill-crown of the nudibranch, *Chromodoris* (Crozier, 1918–19; Crozier and Arey, 1919 *a*, *b*).

It is of interest that small additional weights usually tend to accelerate the speed of spontaneous creeping; while large weights are carried only very slowly, and the slug soon stops creeping. The

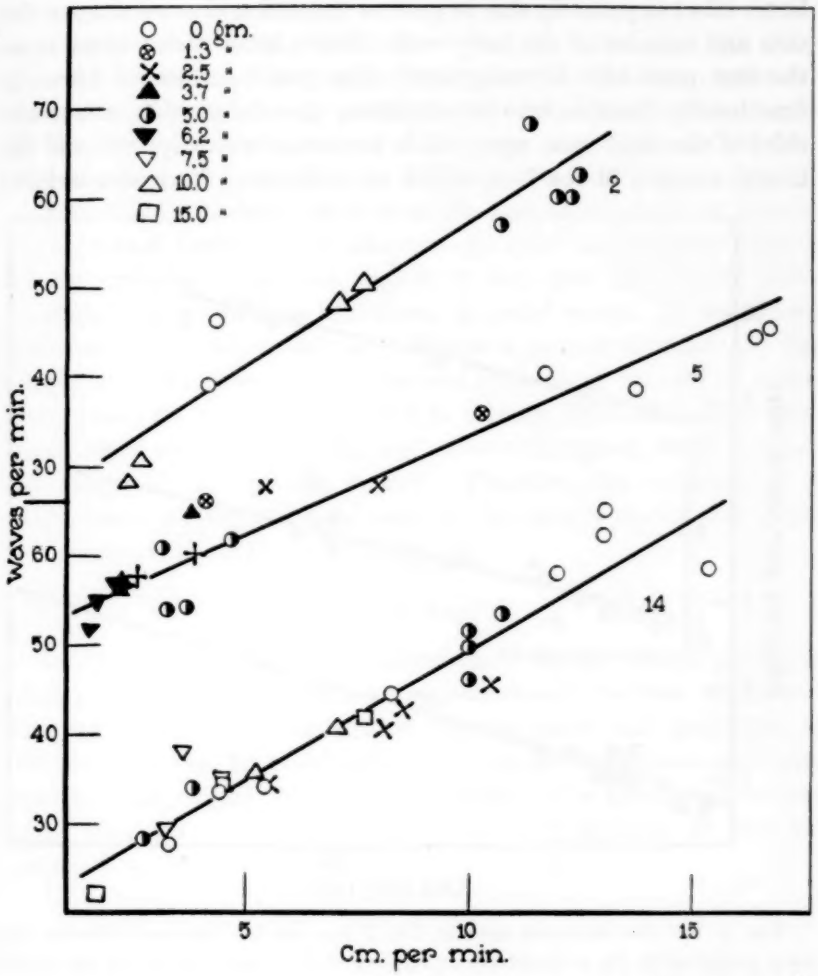


FIG. 1. Typical results illustrating the relation between wave frequency (F) and velocity of creeping (V). The three individuals (2, 5, 14), weighing respectively 9.8, 3.3, and 5.0 gm., were each in different trials loaded with extra weights as indicated. Variation results in part from errors of observation, in part from fluctuations in the velocity with which the individual waves traverse the sole of the foot. Within the limits of this variation the carrying of additional masses does not disturb the relation between wave frequency and speed of progression.

latter effect is probably due in part to the action of tension upon the skin and muscles of the body wall. But a gross direct effect upon the foot must also be recognized. The pedal surface of *Limax* is functionally divisible into two portions, an axial portion, about one third of the total area, upon which locomotor waves appear; and the lateral margins of the foot, which are adhesive. Excessive weights

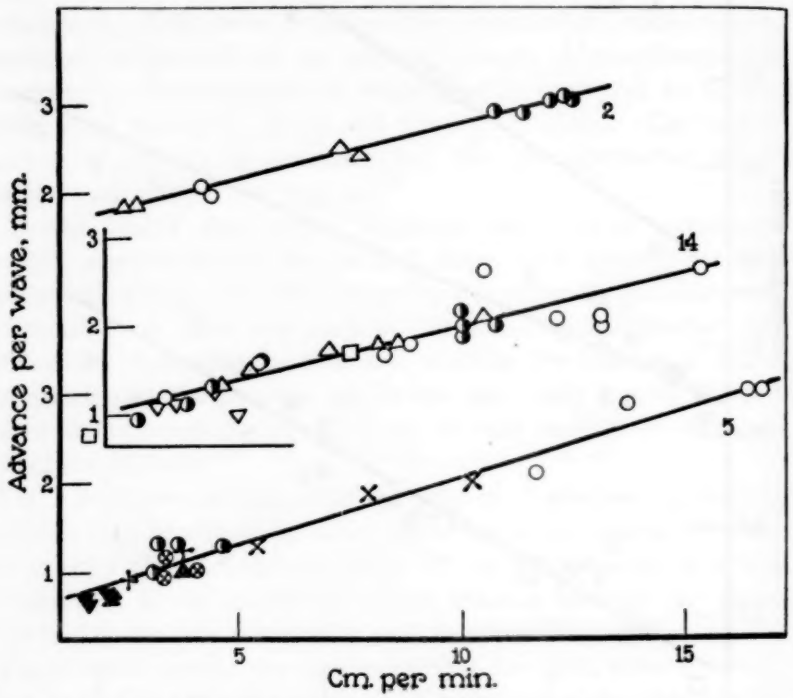


FIG. 2. For the instances used in Fig. 1 the relation between advance due to a single wave (in millimeters) and speed of locomotion is shown not to be disturbed by the presence of added loads.

make adhesion difficult, so that the slug comes to attach more and more vigorously to the glass. After forward motion has ceased, modified pedal waves are still visible on the median area. The modification consists in the suppression of a part of the normal creeping wave. The pedal wave in active progression shows a narrow whitish band succeeded by a darker space (Parker, 1911). When

waves appear which do not move the animal they are white only, lacking the dark band, and they are usually faster than the truly locomotor waves. It is supposed that the white bands represent the "peristaltic" action of only one of the two sets of locomotor muscles in the foot. Incomplete waves of this type may appear on the foot of a stationary slug to which no loads are attached.

Aside from this direct effect upon the foot as an organ of attachment, added loads do not influence the rules for intrinsic activity of the creeping organ, but appear to act upon the central reflex machinery which inhibits or releases the pedal waves. It was shown previously that larger animals evidence a greater advance (A) per single wave, at given temperature and for a given velocity of movement, than do smaller ones. But in a given individual the proportionality of speed to wave size and to wave frequency is not affected by magnitude of the load carried. Therefore, the variation of A with weight of the individual must be due merely to the size of the locomotor elements in the foot.

SUMMARY.

In *Limax* the lifting of additional loads during vertical ascension does not alter the law of linear proportionality between speed and frequency of pedal waves, or that between speed and dimensions of the single waves; for equal velocities of progression these quantities are the same, regardless of the mass lifted. The pedal organ of the slug, although under the control of central impulses, is thus essentially an independent effector.

CITATIONS.

Arey, L. B., and Crozier, W. J., 1919, *a*, *J. Exp. Zool.*, xxix, 157; 1919, *b*, *Proc. Nat. Acad. Sc.*, v, 498.

Crozier, W. J., 1918–19, *J. Gen. Physiol.*, i, 585.

Crozier, W. J., and Arey, L. B., 1919, *J. Exp. Zool.*, xxix, 261.

Crozier, W. J., and Pilz, G. F., 1923–24, *J. Gen. Physiol.*, vi, 711.

Parker, G. H., 1911, *J. Morphol.*, xxii, 155.

TEMPORARY ABOLITION OF PHOTOTROPISM IN LIMAX AFTER FEEDING.

By W. J. CROZIER* and R. L. LIBBY.

(From the Zoological Laboratory, Rutgers University, New Brunswick.)

(Accepted for publication, December 13, 1924.)

I.

The chance observation was made several years ago that the slug *Limax maximus* becomes temporarily non-reactive to light after feeding upon boiled potato. Raw potato did not have this effect. At the time, it was a matter of practical importance merely to avoid complications of this kind while seeking to measure the circus movements of *Limax* as a function of light intensity (Crozier and Cole, 1923). The result of feeding upon boiled potato has been verified upon a number of occasions. We have made a preliminary study of the matter with the idea that it should assist toward an understanding of temporary phases of non-reactiveness to light such as occasionally appear in each slug repeatedly tested. The problem of reversal of phototropism likewise enters, for Frandsen (1901) states that *Limax maximus* is normally photopositive toward light of very low intensities; and we have occasionally found instances of apparent positive phototropism, although we are not quite certain that this is their correct interpretation.

The general idea involved is, simply, that internal variations of the composition of body juices, for example, connected with feeding and nutrition, may determine the exhibition and the direction of phototropic orientation. This notion is of course familiar. Loeb (1889) long ago described the practical loss of heliotropism in the larvæ of *Porthesia* after their first meal. Other definite changes in behavior after feeding may be mentioned, as in connection with the geotropism of *Planaria* (Olmsted, 1917) and in relation to the homing activities of

* Research Associate, Carnegie Institution of Washington.

Onchidium (Arey and Crozier, 1921). The phototropism of *Limulus* seems also to supply a case in point. Cole (1922–23) found these animals positively phototropic when freshly collected, and (1923–24) that during the first 48 hours of laboratory confinement a great increase occurs in the percentage of photopositive responses; at the end of a week, about 50 per cent of the individuals are indifferent to light. This may be related to the fact that in *Limulus* and other forms, according to Morgulis (1922, 1923), brief laboratory confinement suffices to produce notable diminution in the proportion of sugar and of non-protein nitrogen in the blood. In a general way the interpretation of such facts is probably similar to that of the induction of phototropic behavior by the addition of various agents (CO_2, esters, alkaloids) to the environment of photically indifferent aquatic animals (Loeb, 1906; Moore, 1912). With *Limax* it has been shown (Crozier and Federighi, 1924–25) that strychnine may be used to abolish phototropism for a time. But for the understanding of a clear-cut instance of physiological state brought about by feeding, it has seemed desirable to analyze the consequences of *Limax* eating boiled potato, and to identify as far as possible the particular substance responsible for the effect.

II.

To obtain a rough measure of photic reactivity the animal was allowed to creep upon a moistened sheet of glass under which was a system of circular and radial coordinates on white paper. The slug was induced to creep in a line perpendicular to the path of a beam of light; after it had crept for about 15 cm., thus approaching the center of the observation stage, the light was admitted and the subsequent path of the animal was charted upon small scale record sheets. A number of the animals had one eye-tentacle removed a week or more before the experiments began; with these the light was admitted upon the eye-bearing side. The observations were made in a dark room, in which the slugs were kept in individual glass jars with pieces of moist rotting wood, which contributes especially fungi to the slug's diet.

When illuminated in the way described, *Limax* usually turns sharply at an angle of about 90° away from the light and then travels in the path of the beam. Rarely, an individual fails to respond; such

a slug if again tested after an interval usually reacts diagrammatically. The light intensity used in these tests was approximately 200 m.c. Sometimes a higher intensity is required to call forth the sharpest orientation. These are but aspects of a fluctuating state of phototropic responsiveness such as any animal exhibits. The feeding experiments with *Limax* suggest a means of their interpretation.

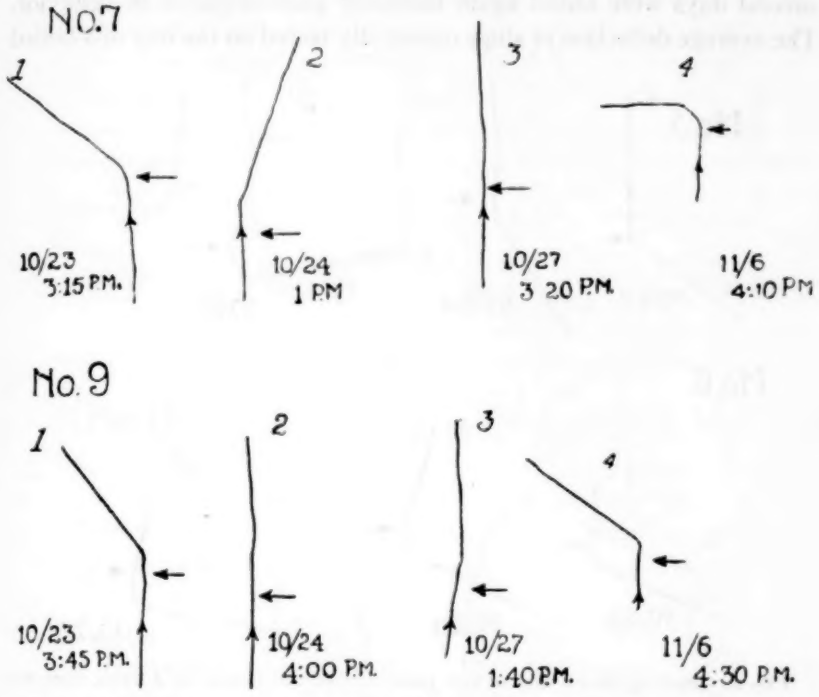


FIG. 1. Tracings of records obtained with two slugs (Nos. 7 and 9) before (*1*) during (*2*, *3*) and after recovery from (*4*) a diet of cooked potato. The potatoes were boiled for 15 minutes. The arrows indicate the direction of illumination and the point on the path at which light was admitted. Scale 1:10.

Typically illustrative experiments serve to indicate the nature of the results. Fig. 1 contains graphic records of the behavior of individuals No. 7 and No. 9. On October 23rd their phototropism was tested, and found decided and negative. When tested in this way, with horizontal light, slugs with but one eye-tentacle may not react with such diagrammatic completeness (*i.e.* 90° turn) as do normal

ones. They were then put in jars each with a boiled potato, of which they ate considerable quantities. On the next afternoon these slugs were indifferent to light, or even in some trials (of which one is given) slightly photopositive. They were left with the potatoes, and ate further; next day the tests were repeated, with the same result. They were then removed to jars provided with bits of rotting wood, and in several days were found again normally photonegative in behavior. The average deflection of slugs repeatedly tested on the day of a boiled

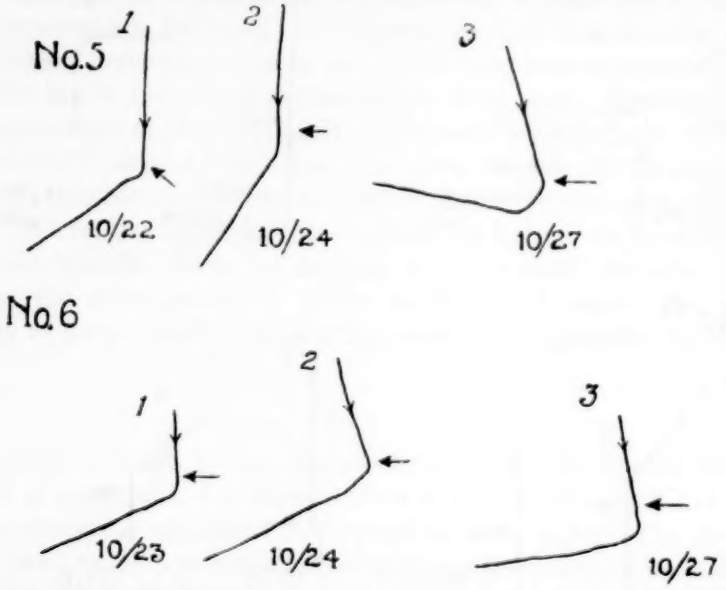


FIG. 2. Showing that a diet of raw potato, even continued for 5 days, does not suppress phototropism.

Individuals 7, 9, 5, and 6, had one eye-tentacle removed. Scale 1:10.

potato meal was 3.7° away from the light. The tests were all made at the same time of day. Raw potato is eaten just as readily as cooked, but does not result in the disappearance of phototropism. Typical results are shown in Fig. 2. The mean photonegative deflection of slugs fed on rotting wood was 54°, of those fed on raw potato 50°.

Suitable control tests showed that the skin of the potato was not concerned with these effects; that other (raw) foods, such as cabbage or apple, did not interfere with phototropism; and that starvation (for as much as 3 weeks) does not materially influence phototropism.

III.

The fecal masses deposited by slugs which have eaten raw potato are whitish, firm, and bulky. They contain quantities of undigested starch grains. Fecal masses from *Limax* fed on boiled potato are much smaller, more slimy, and contain relatively few starch grains. (Data on the amolytic activity of the digestive secretions of pulmonates

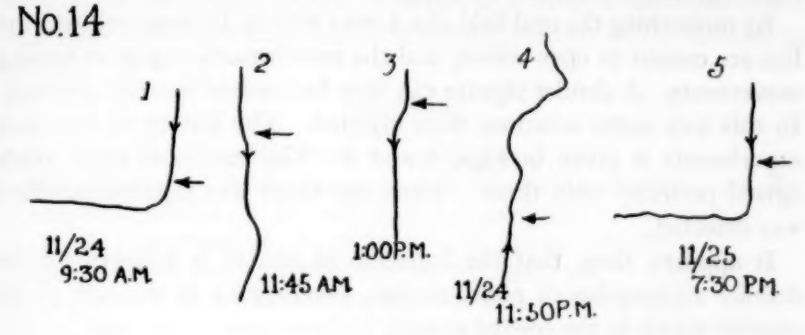


FIG. 3.

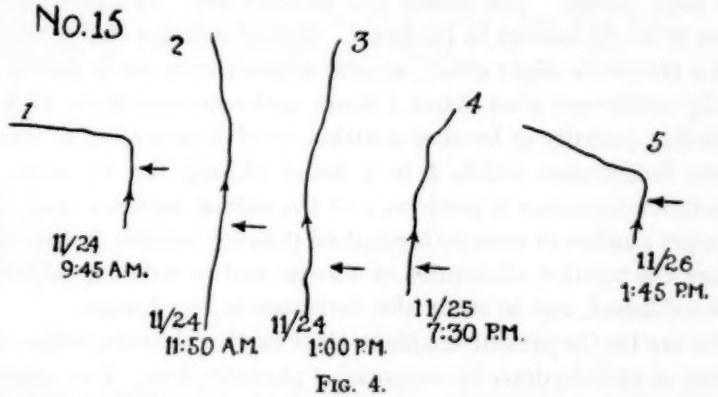


FIG. 4.

FIGS. 3 and 4. The effect of injecting 0.3 cc. of M/4 sucrose solution into the stomach of *Limax* No. 14. This animal had both optic tentacles intact. Trail *1*, before injection; at 11:15 a.m. the sucrose solution was put down the esophagus, and 30 minutes later Trail *2* was obtained. Trails *3* and *4* show continuance of non-reactivity until 11:50 p.m. (tests were made at intervals of several hours); by 7:30 p.m. the next day negative phototropism was fully recovered.

A duplicate experiment, with Slug 15, is detailed in Fig. 4. The effect lasts about 1½ days. Scale 1:10.

is reviewed by Jordan (1913).[1] It was assumed that the influence of cooked potato on phototropism might be due to an effect of carbohydrate metabolism. Cooked potato starch, and corn-starch, was fed to slugs previously starved for several days. At most $\frac{1}{4}$ teaspoonful was all that was eaten. No definite influence on phototropism was apparent, but this may be due to the fact that not much starch was ingested; moreover, tests were not made until 12 hours after feeding.

By moistening the oral field of a *Limax* with M/10 sugar solution the lips are caused to open widely and the mouth parts engage in feeding movements. A slender pipette can then be inserted into the stomach. In this way sugar solutions were injected. The history of two such experiments is given in Figs. 3 and 4. The results of other trials agreed perfectly with these. When tap water was injected no effect was detected.

It appears, then, that the ingestion of sucrose is followed by the definite suppression of phototropism, precisely as in the case of digestible starch in the cooked potato.

In order to complete the proof, sugar solutions were injected into the body cavity. The needle was inserted near the caudal keel, in order to avoid damage to the heart. Sucrose solution (0.5 cc. of M/10) had a relatively slight effect; several animals were made almost photically indifferent after about 2 hours, and remained so for 15 hours. A similar quantity of levulose solution, or of dextrose, led to complete photic indifference within 2 to 4 hours (during the 1st hour after injection much slime is secreted, and the animal does not creep well). A larger number of animals is required than is available at this season before the relative efficiencies of sucrose and of reducing sugars can be established, and to study the variations in blood sugar.

We are for the present unable to trace further the mechanism of the action of carbohydrate in suppressing phototropism. Two points we expect to study are (1) the possible decreased alkalinity of the body juices (or, locally, of nerve centers?) during sugar consumption; and (2) the possibility of reversing the effect.

[1] Jordan (1913), p. 298.

SUMMARY.

When fed upon *cooked* potato the slug *Limax maximus* becomes for a day or more indifferent to light—its natural negative phototropism is suppressed. This effect is not produced by raw potato or other diets; it can be duplicated by injection of sugar solutions into the stomach or into the body fluids, and seems to be due to sugar absorbed during the digestion of the cooked starch. The fact is of interest particularly for the suggestion which it affords as to the explanation of fluctuations in conduct.

CITATIONS.

Arey, L. B., and Crozier, W. J., 1921, *J. Exp. Zool.*, xxxii, 443.
Cole, W. H., 1922–23, *J. Gen. Physiol.*, v, 417; 1923–24, *J. Gen. Physiol.*, vi, 295.
Crozier, W. J., and Cole, W. H., 1923, *Anat. Rec.*, xxvi, 362.
Crozier, W. J., and Federighi, H., 1924–25, *J. Gen. Physiol.*, vii, 221.
Frandsen, P., 1901, *Proc. Am. Acad. Arts and Sc.*, xxxvii, 185.
Jordan, H., 1913, Vergleichende Physiologie wirbelloser Tiere, Jena, i.
Loeb, J., 1889, Der Heliotropismus der Tiere, Wurzburg; 1906, *Arch. ges. Physiol.*, cxv, 564.
Moore, A. R., 1910–15, *Univ. Calif. Pub., Physiol.*, iv, 185.
Morgulis, S., 1922, *J. Biol. Chem.*, l, p. lii; 1923, *J. Biol. Chem.*, lv, p. xxxvi.
Olmsted, J. M. D., 1917, *J. Animal Behavior*, vii, 81.

CRITICAL THERMAL INCREMENTS FOR RHYTHMIC RESPIRATORY MOVEMENTS OF INSECTS.

By W. J. CROZIER and T. B. STIER.

(From the Zoological Laboratory, Rutgers University, New Brunswick, and the Carnegie Institution of Washington.)

(Accepted for publication, December 15, 1924.)

I.

A survey of the critical thermal increments which could be deduced for rhythmic neuromuscular phenomena among arthropods led to the finding that in all but two instances, among those available, the critical increment or temperature characteristic was sensibly identical, namely 12,200 calories (Crozier, 1924–25, *a;* Crozier and Federighi, 1924–25, *b*, *c*). The empirically derived values of the quantity μ in the Arrhenius-Marcelin-Rice equation

$$K_2 = K_1 e^{\frac{\mu}{2}\left(\frac{1}{T_1} - \frac{1}{T_2}\right)}$$

were:

Velocity of progression, *Parajulus**[1]	12,250
" " " ants*	12,220
" " " ants†	12,200 ±
Frequency of chirping, tree crickets*	12,200
" " flashing, fire-flies*	12,200
" " heart beat, *Limulus**	12,000
" " " " *Ceriodaphnia*‡	12,100
" " " " *Bombyx* larvæ§	12,200

The surprising degree of quantitative concordance evident in this list led to the assumption that not only do such rhythmic activities reflect the determination of the frequency of neuromuscular movements through the agency of chemical transformations, but that

[1] Values marked * were derived in a preceding paper (Crozier 1924–25, *a*); †, data from Shapley (1924); ‡, data from Robertson (1906); §, Crozier and Federighi (1924–25, *c*).

the *governing* chemical process must be of identical type in the several instances—at least in the sense that there is involved a common catalyst.[2] The conclusion is the more permissible because the activities compared may be taken to involve "central nervous discharge"[3] as controlling element, and are thus truly homologous.

The rhythmic abdominal movements of respiration, however, in larvæ of *Libellula*, in *Dixippus*, and in other insects, fail to exhibit

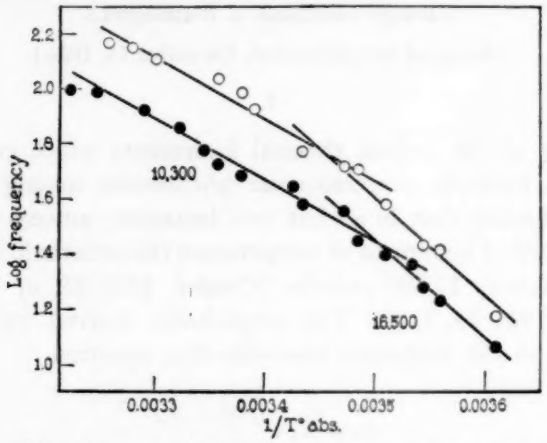


FIG. 1. Breathing rhythm of *Libellula* larvæ at various temperatures (data of Babák and Roček, 1909); black circles, well supplied with oxygen; white circles, under oxygen deficiency. Respiratory frequency is enhanced at low oxygen tensions, but within the limits of variation in the data, the temperature characteristics are unchanged. The increment assigned for the upper range of temperatures is probably too low, owing to the observations above 32°, at about which temperature there may occur destructive effects. There is a suggestion that the critical temperature, at which change of μ occurs, is shifted by oxygen deficiency.

the critical increment 12,200. The probable analytical utility of the method of critical increments makes it desirable to examine carefully the nature of these exceptional instances. The results of such examination, in part contained in the present paper, afford some interesting confirmations of the proposition that analysis

[2] The physicochemical foundation for this view is given in articles by F. O. Rice (1923, 1924).

[3] Evidence that the increment 12,200 probably reflects activity of nerve centers and not of muscles was previously discussed (Crozier, 1924–25, *a*).

through temperature characteristics may ultimately permit the identification of reactions in living matter (Crozier, 1924, 1924–25, *a*, *b;* Crozier and Pilz, 1923–24). They also provide evidence for the validity of the conception of critical temperatures, at which control of a given activity may change from one fundamental reaction to another (Crozier, 1924–25, *a;* Crozier and Federighi, 1923–24, 1924–25, *b*).

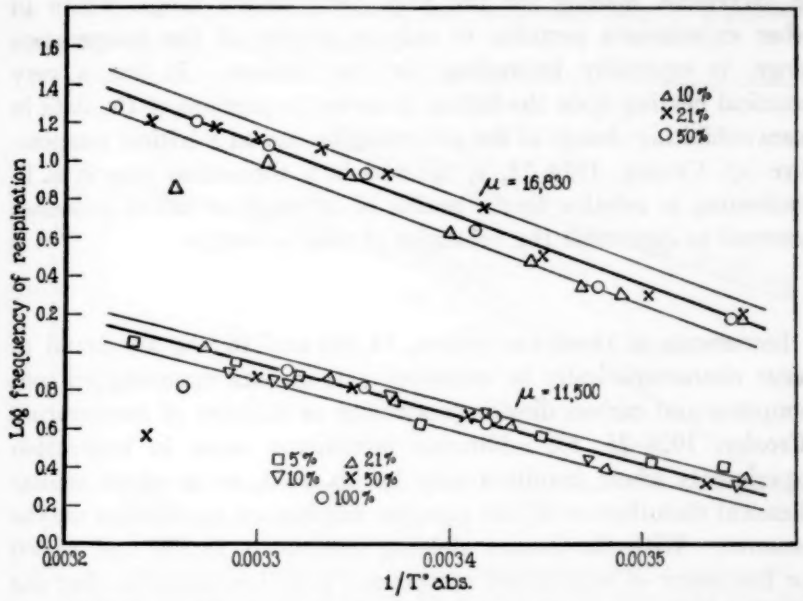


FIG. 2. Two sets of determinations of respiratory frequency in *Dixippus* (von Buddenbrock and von Rohr, 1922–23). Within the limits of variation inherent in the measurements, each set yields a definite but different value of the critical increment, which is consistently maintained in different concentrations of oxygen (percentage O_2 indicated by symbols). Above 32°± C. destructive effects may enter, as shown by three points which depart widely from the rest.

As obtained from data published by Babák and Roček (1909) and by von Buddenbrock and von Rohr (1922–23) the critical increments for respiratory rhythm, in *Libellula* larvæ and in *Dixippus*, are 16,500 and 16,800 calories, respectively (Figs. 1 and 2). In the former case, at higher temperatures μ = 10,300±. A complication

enters when it is found that under certain circumstances, not easily ascertainable from the original accounts, the temperature characteristic for the rhythmic respiratory movements of *Dixippus* turns out to be 11,500±, not 16,500 (Fig. 2). These two sets of measurements would perhaps not be conclusive by themselves, in view of the extent of variation, but it will be shown that they are consistent with new experimental results. The occurrence in some experiments of an increment holding for the total temperature range, which in other experiments pertains to only a portion of the temperature range, is especially interesting for two reasons. It has a very practical bearing upon the fitting of curves to portions of the data in cases exhibiting change of the governing process at a critical temperature (*cf.* Crozier, 1924–25, *a, b*); and in a theoretical way it is illuminating in relation to the notion of catenary or linked processes assumed to determine the velocities of vital activities.

II.

Increments of these two orders, 11,300 and 16,500, are found to occur characteristically in numerous sets of data upon oxygen consumption and carbon dioxide production as function of temperature (Crozier, 1924–25, *b*); additional increments occur in respiration experiments where inanition may be involved, or in which similar chemical disturbances of the complex respiratory mechanism maybe assumed. With the insects yielding increments 11,500 and 16,500 for frequency of respiratory movements it is demonstrable that the velocities of oxygen consumption and of carbon dioxide production also yield, under different circumstances, either the increment 11,500 or 16,200 calories (Fig. 3).

This kind of agreement is fairly strong evidence for the conclusion that the frequency of insect respiratory movements is controlled by the velocity of tissue respiration. It is not necessary to suppose, however, that the increments 11,300± and 16,500± correspond to the *only* two processes which may under any conditions be controlling ones in cell respiration. A certain number of cases are known, for example, involving gaseous exchange or activities which may be dependent thereon, in which there appear temperature characteristics approximately 8,000, 21,000, and 24,000 (Crozier, 1924–25, *b*:

Glaser, 1924–25). The mechanism of cell respiration, as thus far understood (*cf.* Meyerhof, 1924), comprises successive linked processes more numerous than are required to accomodate the several critical increments found associated with respiratory exchanges, if one wishes to assume that these have reference to diverse steps in the intermediary history of the respiratory substrate. These facts complicate the problem of deciding in what particular way the velocity of a given vital process (*i.e.* in morphogenesis) may be dependent upon the energy-yielding reactions.

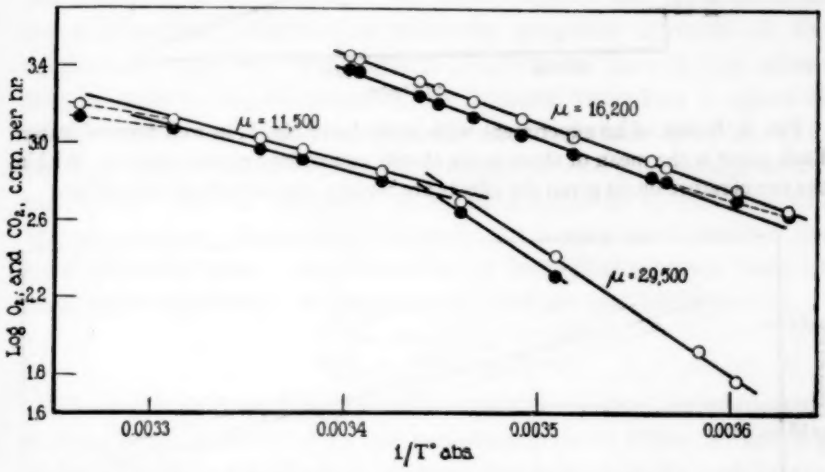


FIG. 3. Upper graphs, velocities of oxygen consumption (white circles) and of CO_2 production (black circles), lower graphs in *Dixippus*, corresponding data from an independent experiment (data of von Buddenbrock and von Rohr, 1922–23). The intersection occurs in the neighborhood of 17°. The deviations indicated by dashed lines may be real and significant.

That the difference between respiratory rhythms yielding respectively $\mu = 11{,}500\pm$ and $\mu = 16{,}500\pm$ is a real difference, and not an artifact of curve fitting, is shown by observations we have made with aquatic larvæ of the dragon fly *Anax*. Two illustrations are given in Figs. 4 and 5. We shall demonstrate presently that the nature of inherent variation in frequency of respiratory movements, at constant temperature, is such as to demand for treatment numerous observations at close temperature intervals. Within the tem-

perature limits used the effect of change in temperature is completely and very rapidly reversible.

In these experiments the animal is near the bottom of a tall beaker of water immersed in a large volume of water of which the tempera-

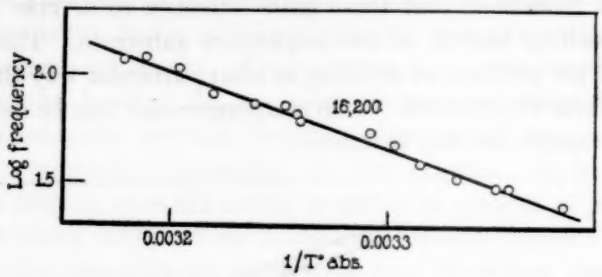


FIG. 4. Result of an experiment with *Anax* larva No. 3, in well aerated water. Each point is the mean of three to six closely concordant measurements. Within the temperature limits given the effects are rapidly and completely reversible.

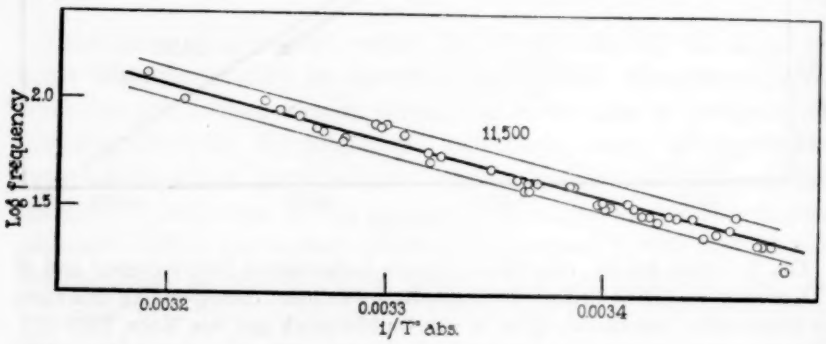


FIG. 5. Data from an experiment with *Anax* larva No. 5, of the same antecedents and history as No. 3 (Fig. 4), but with the animal in stale water (pH about 6.5). Each plotted point is the mean of three to six concordant readings. The extent of variation at each temperature tends to be a constant fraction of the associated mean frequency, so that, as in previous illustrations, the observations occupy a band of constant width. The right-hand terminal point is known to be doubtful.

ture is under control. A clamp may be adjusted upon the thorax of the larva in such a way as to hold the animal in vertical position with the posterior end upward; or the head of the larva may be inserted in a snugly fitting short rubber tube held vertically by a glass

rod. Confinement in this way for as much as 2 days does not affect the character of the rhythmic movements of the rectal respiratory pump. The rubber tube about the eyes eliminates effects due to optic stimulation. With a strong light placed to one side of the thermostat the frequency of the rectal pulsations may be timed very accurately, under a large reading lens, since the open respiratory chamber appears white against the surrounding dark green integument. The light from the lamp passes through an infra-red filter eliminating heat rays, and thus does not affect the temperature of the water or of the animal. We have counted the movements of the anal sclerites which close over the posterior aperture of the respiratory chamber. The figures demonstrate that if due attention be paid to the character of the intrinsic variations in speed of the activity being measured (Crozier and Federighi, 1924–25, *a*, *c*) one may derive values of the critical increments for biological phenomena which rival in precision those obtainable in simple chemical systems; in fact, there might be made out a very good case for the view that the values thus obtained in biological systems may be even more significant for purposes of analysis and identification.

III.

The evidence thus shows that the critical increment for respiratory rhythm of an insect may in one experiment be 11,500±, in another 16,500±. This difference is observed both in aquatic and in terrestrial forms. We have accordingly sought to determine if it is possible to so arrange the experimental conditions that the critical increment for such movements may be altered at will. In terms of the theory underlying these investigations this amounts to the deliberate selection of that particular process of an interrelated series which shall be the slow or controlling one.

It will be shown that it is possible to alter the critical increment for respiratory rhythm, and that the change from one controlling process to another is abrupt, so that one obtains (according to the conditions) one or another of several critical increments without intermediate gradations. The results also substantiate the interpretation previously proposed for cases in which two critical increments are found over different portions of a wide range of tempera-

tures. There is thus provided a definite standpoint from which one may attempt the control of vital processes, in a sense scarcely envisaged hitherto. In general, it may be sought to so alter environmental conditions as to differentially affect the catalytic mechanisms governing velocities of interlinked protoplasmic processes; or by the introduction of foreign materials into the organism one may seek to influence specific reactions. Reciprocally, there is available a possible means of chemically interpreting the mode of action of added catalytic or other agents.

It has been pointed out (Crozier, 1924–25, *a*, *b*) that certain substances, salts and adrenalin for example, may alter the velocity of a protoplasmic activity without changing the value of its temperature characteristic. This is taken to mean that the mechanism for production of the governing catalyst is so affected as to increase or to decrease its total velocity—*i.e.* the amount of catalyst is changed —but that the rate at which active molecules of catalyst are formed bears the same relation to the temperature as before the introduction of the salt or other substance. We now describe experiments in which it appears that of several interconnected processes concerned in the determination of the frequency of rhythmic movements in the grasshopper the respective velocities may be so affected by changed conditions that the normal or typical governing process is no longer in control.

The respiratory rhythm of grasshoppers, *Malanoplus femur-rubrum*, was measured under conditions which permitted a considerable degree of precision in estimations of the frequency of movements. Observations were made during October and November with freshly collected animals of both sexes, and with individuals kept for some days in terraria with growing grass. Each experiment consisted in timing the abdominal movements of one individual at short intervals over a considerable range of temperature. To accomplish this the animal was grasped with broad-tipped forceps on the wing covers, and so held at the center of a stoppered flask immersed in a large jar of water. With the legs removed from contact with a substratum spontaneous movements are reduced. The temperature of the water bath was controlled by addition of small quantities of water from a heated or cooled reservoir. The added water entered by way

of many fine openings in a coil of copper pipe. The temperature could be held constant in this way within 0.1°C. for periods of an hour, and yet rapidly changed. In the different experiments the average rate of heat change was 0.1° per 10 minute interval; this change was made in steps, the temperature being held constant during a measurement. The time required for ten respiratory movements was taken with a stop-watch, and the frequency calculated. A large reading lens, immersed in the thermostat, increased the ease of observation.

There may be distinguished in a practical way three types of respiratory movements in the grasshopper. (For accounts of the mechanism of the respiratory movements, *cf.* Packard (1898), Berlese (1909).) A gentle and very regular type of movement involves the rhythmic rise and fall of the floor of the abdomen. A less common type comprises the rather faint pulsatile activity of the two terminal abdominal segments. At high temperatures, or under oxygen deficiency, and occasionally as result of intrinsic individual circumstances, there occurs a very vigorous peristalsis of the whole abdominal wall. The frequency of this type of movement is less than that for the rise and fall of the ventral surface, but when it occurs continuously at ordinary temperatures its critical increment is the same. The peristaltic kind of movement begins at the anterior end of the abdomen and runs posteriorly; it seems connected with the tracheal circulation of air (Lee, 1924). Beginning at about 30°C., and becoming clearer at temperatures above this, the shallow movements of the abdominal floor by gradations merge into the peristaltic type of breathing. The terminal type of pulsation is most clearly seen with female grasshoppers. Obviously, it is necessary to compare only rates of movements of one type; this has been carefully attended to.

Even with an individual breathing continuously by means of movements of one type, however, the rate of the movements is not constant, but fluctuates in a very definite manner. This is illustrated in Fig. 6. In seeking significant values of the temperature characteristics for such phenomena this type of variation makes it absolutely necessary to secure very numerous readings. The latitude of variation in frequency of rhythm is constant at constant tem-

perature, and as the temperature changes it remains a constant fraction of the mean frequency (unless the critical increment alters, in a new range of temperatures). At lower temperatures its absolute amount is small, and successive stop-watch readings of "time for ten pulsations" therefore agree very closely. At higher temperatures the latitude of variation is higher. This is the kind of organic variation

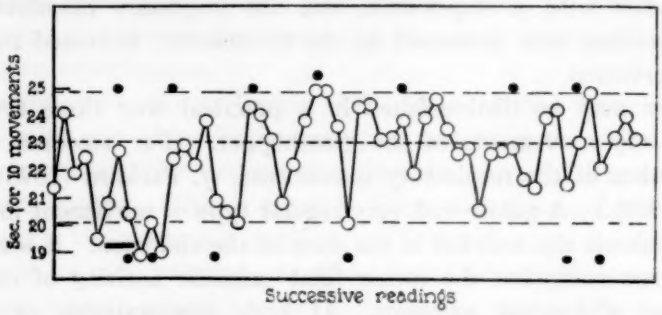


FIG. 6. The time required for ten respiratory movements, in successive groups of ten movements, at constant temperature 32.6°. By the use of two stop-watches no movements escaped enumeration. Since we are interested merely in the kinds of variation to be expected in successive determinations of "time for ten movements," the horizontal axis is divided into equal spaces. When treated from the standpoint of fluctuation in frequency as function of time, the periodic character of the complex tonus rhythms becomes even more apparent. The black horizontal bar indicates a period of defecation. Omitting the readings immediately following this event, it is obvious that, in spite of a very definite rhythmic fluctuation in rate, the extreme readings are confined within a band showing a constant maximal latitude of variation (broken lines). The distribution of the observations is slightly skewed, so that the line of modal frequency (central broken line) is nearer the limit of lower frequencies. It is of interest that the most rapid rates of breathing are coincident with gaping movements of the ovipositor (lower line of black dots), while the slowest rates are coincident with spontaneous movements of one or both anterior legs (upper line of black dots).

already recognized in cell division (*Arbacia* egg, Loeb and Chamberlain, 1915), in connection with the movements of *Oscillatoria*, and in the heart rhythm of silkworms (Crozier and Federighi, 1924–25, *a*,). It may be held to signify regulated fluctuation in the amount of substance (catalyst) which determines the velocity of the controlling process; or else, in more general terms, the result of dynamic balance between several processes implicated in the control of res-

piratory movements. Such fluctuation would be expected to result in a dynamical situation of the sort already assumed on the ground that different critical increments may be associated with a given kind of activity.

IV.

The first experiments were made with freshly collected normal grasshoppers. In order to reduce irregularities accompanying struggling movements due to optic stimulation by movements of

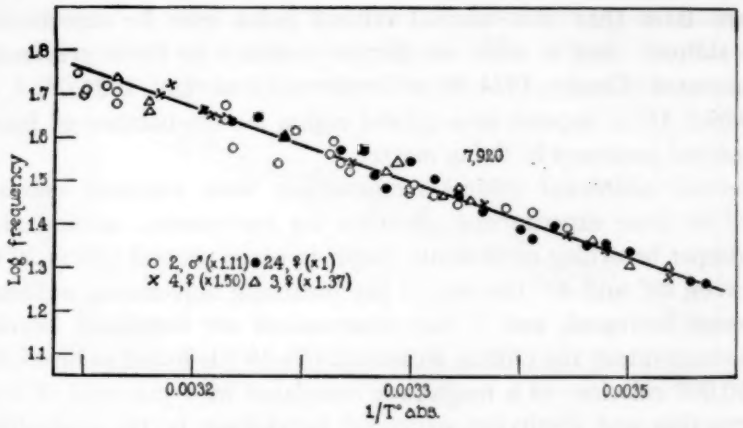


FIG. 7. The critical increment for quiet respiratory movements of normal grasshoppers, *Melanoplus femur-rubrum*, is 7,920 calories. When movements of the legs were evident, or bendings of the abdomen, the readings were discarded. The range of fluctuating variation is such as to make it desirable to combine observations obtained with numbers of different individuals. The data from each grasshopper are distinguished by a symbol. Every plotted point is the mean of a series of four to ten closely agreeing determinations at the particular temperature, with one animal. The rate of breathing movements differs with the individual, at constant temperature. The several series of determinations are brought together by multiplying each by a factor, which in the log plot moves the observations up or down on the vertical axis, but does not change the slope of the line fitting them. There is no constant difference in rate associated with sex. The observations with normal grasshoppers are somewhat more erratic than those with decapitated ones (*cf.* Fig. 8), and the range of variation is perceptibly greater at temperatures above 30°C. (due to greater frequency of spontaneous movements).

the observers, the animal was surrounded by a yellow paper cylinder in which a peep-hole had been cut opposite the abdomen. The

yellow paper admitted sufficient light to permit accurate timing. A small amount of water was present at the bottom of the chamber containing the insect.

With different individuals the determined points are found to be closely coincident (several series are plotted in Fig. 7). It is of especial interest that rhythmic respiratory movements do not appear below 15°±C. Whether the animal be warmed up from a lower temperature, or cooled down to below 15°, this temperature (14.5–16.0° in different instances) is a definite critical point. It will be shown later that this normal critical point may be experimentally shifted. But it adds one further instance to those previously enumerated (Crozier, 1924–25, *a* Crozier and Federighi, 1924–25, *b*, *c*) in which 15°± appears as a critical region for the balance of interconnected processes in living matter.

Several additional critical temperatures were regularly encountered in these experiments. Restless leg movements, accompanied by deeper breathing movements, begin to occur sporadically at 30°C. Between 40° and 43° the rate of the breathing movements suddenly becomes increased, and if the observations are continued beyond this temperature the critical increment (40–49°) is found to be 45,000 to 50,000 calories—of a magnitude associated with processes of heat destruction and signifying structural breakdown in the controlling tissues. In decapitated animals also spontaneous kicking motions frequently become evident for the first time as the animal is brought to 30–31.5°, and again at 39–41°; at the latter temperature sharp movements of the ovipositor, which may or may not accompany an act of defecation, tend to be exhibited.

The temperature characteristic obtained from the data in Fig. 7, and from other similar cases not plotted, is 7,920 calories. This value differs sharply from those previously cited for breathing rhythm in other insects. But turning to Bodine's (1921) measurements of CO_2 production at different temperatures by this particular species of grasshopper, we subsequently found that the CO_2 output increased very little between 0° and 15°, while at about 15° there begins a sharp increase in velocity of CO_2 excretion. From measurements at 15°, 25°, and 38° we find $\mu = 7{,}710 \pm 700$ calories. There is thus, again, a very striking agreement between respiratory movements on

the one hand, and on the other the velocity of respiratory processes, in their relation to temperature.[4]

We then used animals decapitated shortly before the beginning of an experiment. It is known that the head ganglia are not necessary for respiratory movements, but that the abdominal breathing motions are segmentally controlled (Ewing, 1904; Walling, 1906). The blood of *Orthoptera* coagulates readily, so that a seal is formed over the cut. Beheaded animals sometimes live almost as long (at least at this season) as do normal ones in a moist atmosphere but deprived of food (*i.e.* 3 to 6 days).[3] For some typical instances the measurements are plotted in Fig. 8. The critical increment is

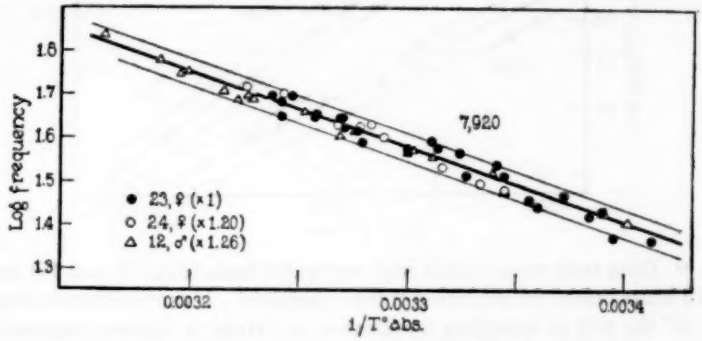


Fig. 8. Data similar to that represented in Fig. 7, but obtained with decapitated animals (experiments begun 1 to 4 hours after decapitation.) The rate of breathing movements is slightly accelerated after several hours subsequent to decapitation, but the critical increment is the same as with normal animals; μ = 7,920. This is true regardless of sex, and is demonstrable in the records from single individuals studied before decapitation and after (*cf.* No. 24, which appears also in Fig. 7).

7,900, agreeing well with that secured in the case of the normal animals. The decapitated individuals are more satisfactory to work with because less subject to spontaneous brief irregularities, and because they did not require to be shielded from visual stimulation; this made it possible to mount a sheet of milk-glass behind the flask containing the grasshopper, so that, through the lens, very precise reckoning of pulsations became possible.

[4] Data from experiments by Batelli and Stern (1913), the interpretation of which may be doubtful because of the high temperatures involved, yield consistently the increment 8,000± (30–40°C.).

V.

If a grasshopper be decapitated and then kept for some hours in a moist atmosphere before being used for the experiment, the inner control of breathing frequency is found altered. The critical increment is then seen to be 16,200 or 11,200, not 7,900 (Fig. 9). Thus, processes characterized by these increments, previously masked, are revealed following decapitation; and it is especially significant

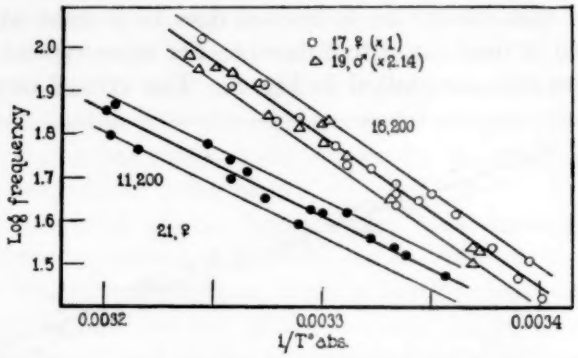


FIG. 9. Data from experiments with two individuals (Nos. 17 and 19) decapitated 15 hours before the beginning of the experiment. If exposed to temperatures above 36° the rate of breathing movements, on return to a lower temperature, is found permanently lowered. The processes involved in decreasing vitality lower the temperature at which thermal destructive effects are induced. In the region of reversible temperature effects, μ = 16,200 to 16,600 (in different experiments).

An experiment with Grasshopper 21, begun 19 hours subsequent to decapitation, shows μ = 11,200.

that these particular thermal increments should be the ones to be evidenced. The (presumably central nervous) mechanism which controls the speed of breathing movements among insects might on this basis be pictured as comprising at least three steps,

$$\underset{1}{A} \rightarrow \underset{2}{B} \rightarrow \underset{3}{C} \rightarrow D,$$

in which the rate of formation of *D* determines the frequency of the movements. Under normal conditions Reaction *1*, say, with Increment 7,900 would then be the slow process, in *Melanoplus*, though

concealed in the other insects studied; under the conditions of gradual exhaustion brought about in the decapitated animal Reaction *2*, or again *3*, with increments 16,500 and 11,500, would successively become the slow, determining, reactions. The latter (μ = 11,500) is possibly an OH′ catalysis, the former (μ = 16,500) may be an iron-catalyzed cell oxidation (Crozier, 1924–25, *b*). It is not unreasonable to suppose that the progressive changes leading to death should, at the last, decrease the local OH′ concentration to a point where its catalytic rôle would be in a limiting position. Since absence of food is known to lead to the death of uninjured *Melanoplus* within a relatively short time, these animals are particularly suitable for such experiments.

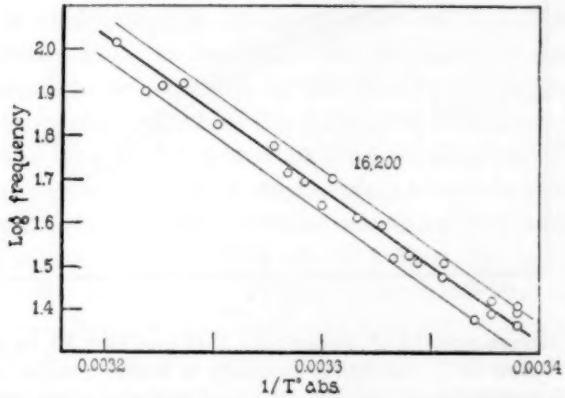


FIG. 10. Grasshopper 20, decapitated, had the two terminal abdominal segments amputated at the same time. The experiment began 1 hour later. The critical increment is 16,200 calories.

We are not rigidly committed, however, to this particular schematic outline. It may be that there are involved reactions not chemically linked but with different loci, spacially distinct. All one need be convinced of is, that several processes forming a catenary series necessary for respiratory rhythm have different temperature characteristics. To get some light upon this we have used (decapitated) animals from which the two posterior segments of the abdomen have been amputated. This was done under the impression that respiratory rhythms with increment other than 7,900 might originate in a different

portion of the ventral nerve chain. One such experiment is recorded in Fig. 10; the temperature characteristic is 16,200. The result of course does not solve the question proposed, but it shows that a freshly decapitated animal which has suffered additional injury may behave like an individual kept for some time following decapitation.

The real proof that consecutive processes with different thermal increments are concerned in the control of breathing rhythm is found in experiments yielding results of the kind illustrated in Fig. 11. The presence of two controlling processes, with gradual shift of the critical temperature, is unmistakable. It is noticeable, also, that

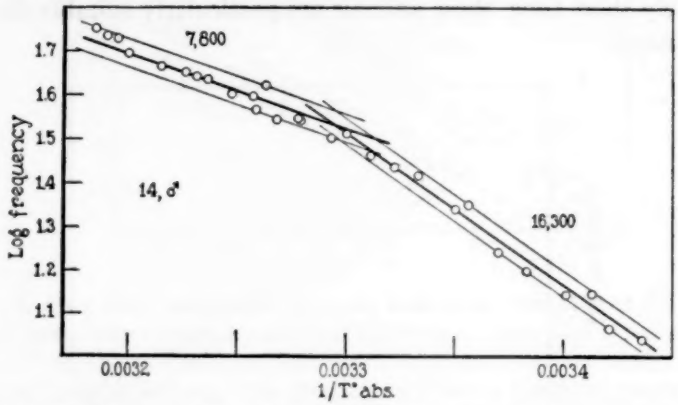


FIG. 11. Grasshopper 14, ♂, decapitated 5:45 p.m. Oct. 30; experiment begun 9:30 p.m. Below 30°C. the critical increment is 16,600; a rather sharp break in the graph is perceptible at 30°, the increment at temperatures above 30° being approximately 7,800. The three left-hand terminal points are less reliable than the others because of a tendency to exhibit kicking movements.

the lowest temperature for consistent rhythm is progressively advanced depending on the time since decapitation. There seems no fruitful way of understanding these results other than with the aid of the conception that the velocities of vital processes are determined by the velocities of dynamically linked systems of chemical transformations, differentially affected by conditions which lower vitality. The absolute magnitude of the pulsation rhythym shifts in a manner consistent with this view. As starvation progresses the frequency is at first increased (Figs. 9 and 10) then decreases.

This kind of interpretation is also consistent with the progressive shift of the minimum temperature for rhythmic breathing movements which is observed subsequent to decapitation. The normal *Melanoplus* initiates continuous rhythmic movements at 14–16.5°; this critical temperature obtains also with decapitated animals for about 4 hours; thereafter the critical temperature gradually moves upward—after 6 hours, 17–18°; after 15 hours, 19°; after 18 hours, 22.5°; after 19 hours, 25.0°. This applies to animals kept at room temperature; we have not yet studied the effect of temperature upon the process of exhaustion itself.

The mechanics of respiration in animals dependent upon a tracheal system are such as to suggest that oxygen supply is likely to be very commonly a limiting condition for respiration. The acidity of the hemolymph of some insects is also consistent with this view. It is not so surprising, then, to find that the frequency of respiratory movements seems to be controlled by the velocity of reactions which control cell respiration. Each of the three critical increments found for these movements, 11,300± and 16,500± in *Libellula*, *Dixippus*, *Anax*, *Melanoplus*, and 7,900 in *Melanoplus*, is represented among those associated with respiration in these insects and in a wide variety of other organisms (Crozier, 1924–25, *b*). That the temperature characteristics for respiratory rhythms differ from the one associated with other rhythmic neuromuscular activities of insects (movements of heart and of appendages) may signify merely that the respiratory movements are controlled by a different group of neurones, with metabolic processes adjusted in a slightly different way. Although the breathing movements are determined by abdominal ganglia, and progression movements (in ants) by thoracic ganglia, this is not the sort of distinction in view; because the contraction of the cardiac aliary muscles of the *Bombyx* caterpillar, providing μ = 12,300 as critical increment, are also mediated by abdominal segmental neurones. There is sufficient evidence, however, to suggest the existence of a definite group of neurones, metabolically distinct from those involved in progression movements for example, which form a kind of "respiratory center" (plurisegmental). We propose later to compare with this breathing rhythm of insects the temperature characteristic for respiration in vertebrates.

VI.

SUMMARY.

The rhythm of abdominal respiratory movements in various insects, aquatic and terrestrial, is shown to possess critical increments 11,500± or 16,500± calories (*Libellula*, *Dixippus*, *Anax*). These are characteristic of processes involved in respiration, and definitely differ from the increment 12,200 calories which is found in a number of instances of (non-respiratory) rhythmic neuromuscular activities of insects and other arthropods. With grasshoppers (*Melanoplus*), normal or freshly decapitated, the critical increment is 7,900, again a value encountered in connection with some phenomena of gaseous exchange and agreeing well with the value obtained for CO_2 output in *Melanoplus*. It is shown that by decapitation the temperature characteristic for abdominal rhythm, in *Melanoplus*, is changed to 16,500, then to 11,300—depending upon the time since decapitation; intermediate values do not appear. The frequency of the respiratory movements seems to be controlled by a metabolically distinct group of neurones. The bearing of these results upon the theory of functional analysis by means of temperature characteristics is discussed, and it is pointed out that a definite standpoint becomes available from which to attempt the specific control of vital processes.

CITATIONS.

Babák, E., and Roček, J., 1909, *Arch. ges. Physiol.*, cxxx, 477.

Battelli, F., and Stern, L., 1913, *Biochem. Z.*, lvi, 50.

Berlese, A., 1909, Gli insetti, Milan, i.

Bodine, J. H., 1921, *J. Exp. Zool.*, xxxii, 137.

von Buddenbrock, W., and von Rohr, G., 1922–23, *Z. allg. Physiol.*, xx, 111.

Crozier, W. J., 1924, *Proc. Nat. Acad. Sc.*, x, 461; 1924–25, *a*, *J. Gen. Physiol.*, vii, 123; 1924–25, *b*, *J. Gen. Physiol.*, vii, 189.

Crozier, W. J., and Federighi, H., 1923–24, *Proc. Soc. Exp. Biol. and Med.*, xxi, 56; 1924–25 *a*, *J. Gen. Physiol.*, vii, 137; 1924–25, *b*, *J. Gen. Physiol.*, 151; 1924–25, *c*, *Proc. Nat. Acad. Sc.* (in press).

Crozier, W. J., and Pilz, G. F., 1923–24, *J. Gen. Physiol.*, vi, 711.

Ewing, H. Z., 1904, *Kansas Univ. Sc. Bull.*, ii, 305.

Glaser, O., 1924–25, *J. Gen. Physiol.*, vii, 177.

Lee, M. O., 1924, *Am. J. Physiol.*, lxviii, 135.

Loeb, J., and Chamberlain, M. M., 1915, *J. Exp. Zool.*, xix, 559.

Meyerhof, O., 1924, Chemical dynamics of life phænomena, Monographs on experimental biology, Philadelphia and London.

Packard, A. S., 1898, Text-book of entomology, New York.

Rice, F. O., 1923, *J. Am. Chem. Soc.*, xlv, 2808; in Taylor, H. S., A treatise on physical chemistry, New York, 1924, ii, Chapter XIV, 865. Rice, F. O., Fryling, C. F., and Wesolowski, W. A., 1924, *J. Am. Chem. Soc.*, xlvi, 2405.

Robertson, T. B., 1905–06, *Biol. Bull.*, x, 242.

Shapley, H., 1924, *Proc. Nat. Acad. Sc.*, x, 436.

Walling, E. V., 1906, *J. Exp. Zool.*, iii, 621.